

An agency undermined by silence

The National Science Foundation has found itself stranded by the US budgetary battles, while other, more contentious agencies know what they will have to spend. Scientists and science's beneficiaries need to sharpen their political wits.

FOR science agencies in the United States, the current budget wars have come to resemble a frantic game of musical chairs. The music is due to stop again at the end of this week, and the National Aeronautics and Space Administration (NASA) and the National Science Foundation (NSF) will be among those scrambling to find a seat.

Four months into the 1996 financial year, neither of the two agencies has a budget. What makes the citizenry a touch cynical about the process is its apparent randomness. The NSF, which funds most non-biomedical university science in the United States, has no enemies to speak of in Washington, but five weeks of government shutdowns since October and the continuing absence of a 1996 budget have thrown its operations into chaos.

By contrast, the Department of Energy, whose operations and, indeed, very existence are subject to fierce political controversy, received a budget last November. Its laboratories — the core of physics research in the United States — have been working normally since then. The National Institutes of Health (NIH), the biomedical research agency, was dragged into the shutdown over Christmas but was dramatically rescued two weeks ago (see *Nature* 379, 101; 1996) and now stands aside from the mêlée with an almost generous budget increase of 6 per cent.

There is a widespread view (yet to be proved) that NASA's close ties with aerospace contractors will ultimately save it from too much further damage. But the people at NSF are beginning to feel isolated, unprotected and angry — as was made vividly clear last week by NSF's director, Neal Lane, speaking at a meeting of the American Astronomical Society. According to him, it could take NSF all year to get back to normal service.

Lane also issued a thinly disguised plea for scientists to mount a far stronger lobby on NSF's behalf, noting the "perceived stony silence of the science and technology community" over the past year — a manifest lack of concern that has not gone unnoticed in Washington. In off-the-record briefings, NSF officials go further, criticizing the powerful university lobby for fiddling over the Christmas holiday while Congress let the NSF burn. And where was the voice of industry?

There is no doubt that the community has been slow to rally behind NSF during this, its latest hour of need. But mitigating factors need to be appreciated. December and January are relatively quiet months for grant renewals. The agency's \$2-billion research budget is spread across all scientific disciplines, so that few of them are entirely dependent on the NSF (ground-based astronomy is one exception) in the way medical schools, for example, are entirely dependent on the NIH. Most universities were able to pay researchers whose grants were held up by the shutdown without placing undue strain on their financial resources.

Additionally, it is hard to see what the community could do for NSF, since no tangible proposal existed (or exists now) for it to rally behind. NIH managed to be rescued from the budget rubble because the Speaker of the House of Representatives, Newt Gingrich, has developed a soft spot for biomedical research, and because the spectre of victims of AIDS and other illnesses being yanked from clinical trials was too much for the public to bear. And NIH needed to be plucked from an appropriations bill — Labor, Health and Human Services and Education — so

chronically bogged down that it may never pass into law.

NSF has neither such special friends nor so emotive a case for special treatment, and the authors of its appropriations bill — the Veterans' Affairs, Housing and Urban Development and Independent Agencies (VA-HUD) bill — still hope to hold their work together, negotiate with the president, and pass something into law.

So the nation's most universally respected science agency limps on without a budget, its staff branded "non-essential" and months behind in their work of selecting and dispensing 20,000 research grants a year, and with the persistent threat of further shutdowns pending.

Lane is prohibited by law from orchestrating support for his own agency, but during the past year other groups — such as the Science Coalition, founded last year by Harvard and a group of other leading research universities — have sought to build support for science on a broad front. Until the latest crisis, they appeared to be having some success, with lawmakers led by Robert Walker (Republican, Pennsylvania), chair of the Science committee in the House of Representatives, showing firm support for the NSF.

Lane refers to the situation since October as "an unprecedented, abominable mess" but nonetheless hopes that it will serve to wake up the community. The normally mild-mannered NSF director says that what he calls "the entire sordid episode" has irreversibly changed the image of public service. He is right to be "very worried" about the implications for NSF as well as other agencies.

There have been many opprobrious adjectives used to describe the budget battle, not just by officials of the Clinton administration, such as Lane, but also by members of a disgruntled public across America. Some sense of proportion should be retained, however: the shutdowns at least represent a real effort by the political class to grapple with tough fiscal problems.

The final outcome of that effort will almost certainly be a plan to balance the budget by 2002, which will involve drastic cuts in discretionary spending over coming years. Science and technology consume one-seventh of that discretionary spending — \$70 billion a year — and will not be immune from the intense and repeated scrutiny which will fall on all government programmes.

So the battle must be joined. Science has a good story to tell. The Consortium for Oceanographic Research and Education (CORE), a research lobby group headed by the former energy secretary, Admiral James Watkins, is showing the way ahead at a joint hearing this week of three powerful House of Representatives committees, for which CORE has been pushing for months (see page 283). Watkins and Lane will join Bruce Alberts, the president of the National Academy of Sciences, Admiral Jeremy Boorda, head of the Navy, and others to explain to Congress why America needs the ocean sciences.

The hearing carries an implicit message for the rest of the scientific community, too. Scientists live off the federal government, but do too little to sustain the system that enables them to do their work. Since the Republican election victory of November 1994, there has been too much hand-wringing in the community, and not enough constructive engagement. □



Ocean researchers make waves in quest for funds and military data

Washington. Ocean scientists are planning to make a direct appeal to Congress this week for a concerted effort to obtain open access to secret ocean data of high scientific value held by the US military, and for an extra \$25 million a year to strengthen university research in their discipline.

In particular, Congress will be asked to back a National Ocean Leadership Council to coordinate ocean research across the government, a proposal that is being put to it by the Consortium for Oceanographic Research and Education (CORE).

The proposal will be put forward by CORE's president, Admiral James Watkins, a former energy secretary under President George Bush. The council would be co-chaired by the top-ranking admiral of the US Navy and the administrator of the National Oceanic and Atmospheric Administration (NOAA).

A priority for the council would be to accelerate the declassification of extensive sets of magnetic and acoustic data on the world's oceans that have been gathered by the US Navy. Watkins says that the existing system for doing this is cumbersome and bureaucratic. "It's a slow system that needs aggressive reappraisal," he says.

CORE's plan is due to be put to a joint hearing of three House of Representatives' subcommittees on ocean research. Witnesses will include Admiral Jeremy Boorda, chief of naval operations, Bruce Alberts, president of the National Academy of Sciences, Neal Lane, director of the National Science Foundation (NSF), NOAA administrator James Baker, as well as Watkins.

The research subcommittee of the National Security Committee, chaired by Curt Weldon (Republican, Pennsylvania), will join with the energy and environment subcommittee of the Science Committee, chaired by Dana Rohrabacher (Republican, California), and the fisheries, wildlife and oceans subcommittee of the Resources Committee, chaired by James Saxton (Republican, New Jersey), for the hearing.

The event has been orchestrated by Weldon and his fellow-Republican Watkins as a boost to ocean research at a time of shrinking research budgets. But some researchers fear that CORE's high-profile tactics could hurt other scientific disciplines.

Some observers say that there is a thin line between speaking out for the discipline and doing so at the expense of others. "Sometimes CORE gets close to that line," says one senior ocean scientist, adding: "I always worry that disciplines that are not so

well-organized will be deemed less worthy."

Watkins declines to suggest where extra money for oceanography should come from. "That's not our responsibility," he says. "Our job is to say this is good stuff, thoroughly peer-reviewed and underfunded."

But he feels that research on the oceans has been overshadowed by other environmental sciences. "The oceans were never mentioned once at Rio," he says, referring to the Earth Summit at Rio de Janeiro in 1992. Richard Spinrad, research director at CORE, goes further, by arguing that oceanography "should not be thrown into the basket with all the other environmental sciences".

According to the Ocean Studies Board at the National Research Council, total federal investment in ocean research has remained relatively constant at \$525 million a year (in 1995 dollars) since 1982. More than a third of this is funded by NSF, and a further third between the Office of Naval Research and NOAA.

But CORE points out that investment in basic science by the federal government has grown from \$8 billion to \$14 billion in that period, and that oceanography's share has therefore fallen from 7 to 4 per cent.

Congressional staff say that, although ocean research has powerful friends in Congress, extra money is unlikely in the present fiscal climate. But they say that there is support for the opening-up of more Navy data. This week's hearing will consider whether legislation is needed to assist in this, and Watkins is pushing for a similar hearing in the Senate, where he has strong support from Mark Hatfield (Republican, Oregon),

Watkins: coordination also needs improving.

chair of the Appropriations Committee.

Civilian oceanographers recently benefited from the release by the Department of Defense of data on the ocean surface, gathered by Geosat (see page 300). But ocean scientists say they could learn more about ocean floor chronology from magnetic field data gathered by military ships and aircraft, as ocean depth soundings would give a more accurate contoured map of the floor than one inferred from satellite data.

The Navy also has a quantity of data on the thickness of sediment on the ocean floor, and on water temperatures beneath the Arctic ice, which it gathered when 'playing hide and seek' with Soviet submarines.

In addition, an array of Navy listening stations known as Sossus — the Sound Surveillance System — collects data to which marine biologists long for access. According to one congressional staff member, biologists "learned more about whales in a few days than they'd learned in the previous forty years" when allowed access to Sossus during a brief test run in 1994. At that time, the Navy was considering transferring Sossus to civilian control, but has since decided to retain it for national security purposes.

The Navy's concern is not that the data themselves would be helpful to an enemy, but that the concentration of data points in unexpected parts of the world would betray US interests. Oceanographers say the data could be processed to prevent this undesired leakage of information.

CORE's proposal of a Leadership Council will not be welcomed by the Clinton administration. According to James Baker of NOAA, mechanisms for coordination already exist, through three subcommittees of the White House's National Science and Technology Council. And, says Baker, NOAA has "a direct and formal tie" with the Navy and the Air Force: senior officers of both forces work in NOAA as advisers to Baker's office.

Colin Macilwain

NASA's planet search programme 'on track'

San Antonio, Texas. The launch of a space-based interferometer capable of detecting life around Earth-like planets should be feasible within ten years, Daniel Goldin, head of the US National Aeronautics and Space Administration (NASA), told a meeting of the American Astronomical Society in San Antonio, Texas, last week.

Goldin's vision for space science includes a 'next-generation' space telescope, and an aggressive programme to search for planets

around other stars (see *Nature* 378, 650; 1995). While warning "I'm not making any guarantees", he said the search should be possible even with NASA's shrinking budget.

Goldin was optimistic about two near-term projects — the Advanced X-Ray Astrophysics Facility (AXAF) and the Space Infrared Telescope Facility (SIRTF). "I will personally throw my body across the tracks if anyone interferes with SIRTF," he told the meeting.

Tony Reichhardt

Europe is warned of a 'neutron drought' ...

Paris. European physicists and other researchers warned last week that the closure of many smaller neutron sources, at a time when the demand for neutron scattering techniques is growing, raises the prospect of a "neutron drought" in Europe, with demand for neutrons outstripping supply around the turn of the century.

The warning emerged from a workshop on nuclear scattering techniques organized by the European Science Foundation (ESF) and the European Neutron Scattering Association in Autrans near Grenoble in France, and attended by 80 leading scientists and engineers. The meeting concluded that an immediate shortage of neutrons can be avoided only by upgrading existing large facilities, and also recommended building a third-generation neutron source in Europe early in the next century.

Neutron scattering techniques are increasingly used by materials scientists, condensed-matter physicists, structural biologists and others studying the structure of molecules, or *in situ* processes such as enzyme transport across membranes. These neutrons are produced either as a steady stream generated by nuclear reactors, or as short pulses produced by accelerating protons into a target made of a heavy metal such as depleted uranium or tantalum — a process known as spallation.

Europe has the world's two most powerful neutron sources, the 58.3-MW reactor at the Institut Laue-Langevin (ILL) in

Megascience Forum. The only new source scheduled to have come on line by this time is the planned 20-MW FRM2 research reactor in Munich, Germany (see below).

At the same time, demand for neutron sources is growing and expected to continue to do so. Neutron scattering is shifting from "an exotic to a routine tool", says Reinhard Scherm, director of ILL. Neutron researchers also argue that a third-generation machine will eventually be needed for studies of more complex materials.

Last week's meeting represents an attempt by the diverse community of neutron users to lobby more effectively for better facilities. The fact that they are "now speaking with a single voice" is important to gaining support for neutron facilities, says Peter Tindemans, the chairman of the OECD Megascience Forum, which is expected to set up a working party on neutron sources at a meeting this week.

The recommendations from the Autrans meeting also reflect a new pragmatism which acknowledges that Europe's cash-strapped research agencies are unlikely to back the building of new facilities in the short term, and that to gain such support, the scientific community must make a more convincing case than in the past.

"We can no longer even begin to contemplate building a new machine until the scientific community has itself made a watertight case for the need for it," says Peter Day, professor of chemistry at the Royal Institution in London, and a member of ESF's working group on physical and engineering sciences. The consensus achieved at last week's meeting by the users of neutron sources themselves is a big step in this direction, he says.

Political support for neutron sources is certainly lacking. Although ILL was recently refurbished at a cost of ECU50 million

(US\$62.5 million), budgetary restrictions caused by a reduced contribution from the United Kingdom, which along with France and Germany is one of the main contributors to ILL, led to the number of beamlines being reduced last year from 31 to 25. Similarly, a proposal by ISIS to install a second target, which would double its capacity, has not yet obtained funding.

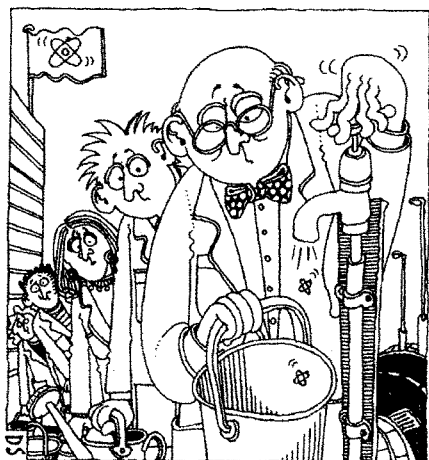
European researchers are keen to maintain their lead over the United States in neutron scattering, one of the few capital-intensive areas of research where this is the case. Europe has three times as many neutron scientists as the United States, and has spent five times more on neutron facilities over the past three decades.

Last year, the United States abandoned work on the \$3-billion Advanced Neutron Source (ANS), which was to have been built at Oak Ridge National Laboratory (ORNL) in Tennessee and would have been five times more powerful than ILL. The Department of Energy subsequently set up a high-level committee to reassess the needs of US researchers for neutron sources.

But the proposals that are likely to emerge will at best allow the United States to catch up with Europe. A proposal to upgrade ORNL, for example, would result in a machine equivalent to ILL. ORNL and the Argonne National Laboratory in Illinois are also candidates for a proposed 1-MW spallation source. Although this would be five times as powerful as ISIS, Europe already has plans for a much larger machine.

This is a proposed 5-MW European Spallation Source (ESS) being designed jointly by the Rutherford Appleton Laboratory in the United Kingdom and the KFA Jülich in Germany, and would be 30 times more powerful than the 160-KW ISIS facility.

Declan Butler



Grenoble and the ISIS pulsed spallation source at the Rutherford Appleton Laboratory in the United Kingdom. (Japan has a 5 per cent stake in ISIS, but itself plans to begin building a machine — Spring-8 — three times more powerful in 1997.)

But both ILL and ISIS are already oversubscribed, while only two of Europe's 19 other existing facilities — the reactor at the Hahn-Meitner Institute (HMI) reactor in Berlin and the Orphie reactor in Paris — will be running at the end of the century, according to a 1993 report of the OECD's

... as leak fuels German uranium debate

Munich. A document leaked from within Germany's federal research ministry (BMBF) has suggested that the government may be reconsidering its support for the construction by the Technical University of Munich in Garching of a neutron source based on a research reactor — known as FRM2 — designed to use controversial highly enriched uranium (HEU).

The memorandum was sent to the research ministry from the federal environment ministry. It describes a telephone conversation in which Anton Axmann, the project leader of the FRM2, apparently admits that the research reactor that provides the neutron source could be redesigned, without significant loss of performance, to burn uranium that is only 70 per cent enriched.

The leak has coincided with a visit to Bavaria of officials from the US Departments of State and Energy, who are trying to persuade both Bavarian government officials and scientists from the university to redesign the reactor to burn low-enriched — that is, less than 20 per cent — uranium (LEU). Fuel elements at the 70 per cent enrichment level referred to by Axmann had been designed by the Argonne National Laboratory for the US Advanced Neutron Source, which was abandoned last year during the planning stage. But US officials say that this intermediate enrichment level would still be of weapons grade, and therefore unacceptable.

The research reactor, if built as now planned, would be the first specifically designed to burn weapons-grade HEU, at ►

93 per cent enrichment, since the signing in 1978 of the international agreement on Reduced Enrichment for Research and Test Reactors (RERTR), aimed at eliminating civilian trade in HEU. Following its signing, nearly all research reactors in Europe have either converted, or agreed to convert, to the use of LEU.

The Munich university, however, is resisting pressure for a redesign — costly in terms of both time and money — and wants to start building the reactor this year. The government has so far backed its stance. But handwritten notes on the leaked memorandum, written by officials of the research ministry, say that Axmann's position "qualifies the BMBF's arguments considerably". Bernd Neumann, the deputy research minister, told parliament last week that the BMBF, which will co-finance the reactor, might reconsider its previous support for the use of HEU in the light of this new information.

The BMBF has no direct influence over the university, as German universities come under individual *Länder* rather than the federal government. But as it has foreign policy implications, the federal government could override Bavaria on this matter.

At last week's meeting, US officials rejected a proposal from the university to convert the reactor to LEU after ten years of operation, if this would have no impact on the reactor's performance. The university believes that it could obtain up to ten years' worth of HEU from stocks currently under the control of Euratom.

US officials said that the reactor must be redesigned immediately — with a larger reactor core — if conversion, which would require an increase in power, is to be technically possible. They confirmed that the United States, currently the West's only supplier of HEU, will not supply such fuel for the FRM2 in future. The Euratom Supply Agency, however, is said to be negotiating with Russia for the supply of fuel (see *Nature* 379, 201; 1996). This has sparked concern among some US congressmen that Euratom appears to be departing from the terms of the nuclear non-proliferation treaty which came into effect in 1970.

Representative Charles Schumer from New York, a Democrat with a special interest in non-proliferation issues, has now written to Warren Christopher, the US Secretary of State, requesting that a \$12-billion agreement under which the United States will buy Russian HEU extracted from nuclear weapons and blend it down to LEU, should be used to stop Russia from selling HEU to Euratom.

Schumer says he will also try to delay congressional approval of the renewal of the US-Euratom agreement — which covers trade in nuclear materials between the United States and Europe, and which expired last month — without a clear indication that Russia will not sell HEU to Euratom.

Alison Abbott & Quirin Schiermeier

Corruption claims hang over Amazon surveillance system

São Paulo. An ambitious US\$1.4-billion radar surveillance system for the Amazon is expected to be approved by the Brazilian government this week, despite opposition from scientists and fallout from a corruption scandal involving government officials and Raytheon, the US company that was awarded the project contract.

Opposition politicians have questioned both the high costs and the legality of the Sistema de Vigilância da Amazônia (Amazon Surveillance System, SIVAM) in the light of the resignation last November of Maura Gandra, the air force minister, over a bribery scandal. Gandra, it has emerged, was friendly with a Raytheon lobbyist alleged to have tried to bribe an opposition senator into supporting SIVAM.

But the government, despite the mounting opposition, is expected to press ahead with its plans to approve SIVAM. President Fernando Henrique Cardoso reaffirmed his support for the project last month, and the senate committee charged with investigating the corruption allegations is bringing forward the announcement of a decision on the project from 7 February to this week.

SIVAM is designed to improve air traffic control and environmental monitoring in the Amazon basin. Managed by the air force, it combines data from remote-sensing satellites and airborne air control radar. But the project has been dogged by controversy since May 1995, when Raytheon's Brazilian partner, Automation and Control Systems Engineering (ESCA), was disqualified from taking part amid allegations of tax evasion.

The recent bribery allegation emerged when the Brazilian press published transcripts of telephone conversations between Júlio César Gomes dos Santos, the presidential head of protocol, and Jose Affonso Assumpcao, a lobbyist and adviser for Raytheon. Santos is alleged to have asked if Assumpcao had "already paid the guy", referring to a senator opposed to the project. Gandra, who was not air force minister at the time, and Santos have both resigned.

Assumpcao has said he cannot recall any such conversation, but assumed that any question about payments must have been "a joke". Gilberto Miranda, an opposition senator from Amazonas state, has denied any wrongdoing, and claims he would immediately report any attempt to buy his influence.

But SIVAM faces further controversy. The government says the scheme will help to monitor illegal activity such as drugs trafficking and river pollution. Scientists and

opposition politicians, however, believe that the project is expensive, unnecessarily complicated and irrelevant, and favour a cheaper alternative incorporating local know-how.

The Brazilian Society for the Progress of Science (SBPC), for example, says Brazil's problems of deforestation, land use and pollution derive from inadequate social, health and education policies and not from a need for a billion-dollar surveillance system.

Scientists also question SIVAM's net value to Brazilian research. They argue that the project largely ignores indigenous capacity in universities, research institutes and industry and complain that even the Nation-

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Burning issue: the Amazon needs effective policies, not an expensive surveillance system, scientists say.

al Institute for Space Research (INPE) has only a minor share in the project, despite providing most of the personnel for SIVAM's remote sensing operation.

"It is a lost opportunity," says Rogério César de Cerqueira Leite, a physicist at Campinas University. Earlier this month, Leite wrote a newspaper article in which he suggested that SIVAM included "obsolete paraphernalia" and was inefficient. "Brazil could produce a more efficient and cheaper system itself," he wrote.

Last week, the committee of the Brazilian Senate set up to investigate the corruption allegations prevented a SIVAM critic, a former director of research and development at the Brazilian air force, from giving evidence. Ivan Frota, a reserve air force general who in 1990 investigated an alternative Amazon surveillance system, which, he says was 42 per cent cheaper than Raytheon's winning bid, was prevented from speaking. He had earlier alleged that the government had been "buying congressmen with favours".

Frota's view that the government has paid too much for SIVAM has since received backing from an opposition politician. Arlindo Chiniaglia, of the Workers' Party in São Paulo, has alleged that Raytheon could be overcharging for the project by as much as 55 per cent. The SBPC, which has assessed Chiniaglia's claims, believes the figure amounts to about 40 per cent.

Ricardo Bonalume Neto

M. Harvey/Panos Pictures

University equipment fund meets with mixed reaction

London. Britain's universities, faced with growing difficulty in maintaining their research facilities, have given a cautious, if slightly sceptical, welcome to a scheme announced by the government last week for competitive grants for new equipment.

Under the scheme, which was announced by Ian Lang, the cabinet minister responsible for science and technology, the government is making available a total of £18 million for two schemes, for equipment costing below and above £250,000 respectively. In both cases, the government grants will have to be complemented by an equal sum raised from private sources.

All applications will be peer reviewed through the research councils. Furthermore, the topics to which the equipment is related will be judged in terms of the extent to which they coincide with the priorities identified in the recent Technology Foresight exercise.

Ian Taylor, the junior minister for science, said after the announcement that the government had launched the equipment grant scheme in recognition of the problems faced by universities. "This shows what can be done by bringing the two sides of the dual support system together," said Taylor, referring to the UK policy of funding university research through both the Department of Education and Employment and the Office of Science and Technology (OST). "I do not want there to be an ongoing funding difficulty for equipment related to research."

But representatives of the university community, while acknowledging that the new scheme will inevitably be of value, claim that the amount of money involved is far below current requirements. "This is a drop in the ocean compared to the real needs," says Michael Powell of the Committee of Vice Chancellors and Principals.

Powell points out that, because only three months after an outcry among university scientists caused by the government's decision to impose a major cut in the funds available to the Department of Education for capital funding grants to universities this year, the announcement of the new scheme "looks a bit like a panic measure".

The single largest contributor to the new scheme will be the Higher Education Funding Council for England (HEFCE), which will provide £11.5 million towards the fund for major items of research equipment costing over £250,000 (a further £1.5 million will be provided by HEFCE's Scottish equivalent).

Brian Fender, the chief executive of the funding council, says that he has been able to find the extra funds by a judicious mix of squeezing and slippage in existing programmes. He adds that the money being

made available to universities will address three separate objectives.

First, it will help "a little" towards meeting the capital investment needs of higher education institutions; secondly, it will be a way of linking universities to the priorities outlined in the Technology Foresight exercise — many of the reports from which, published last summer, identified the state of university equipment as a top priority for the government.



Fender: keen to boost Foresight priorities.

Thirdly, says Fender, the new scheme "has been a good opportunity for drawing the funding councils, the research councils and the OST closer together", a goal already identified in the government's white paper on science published in 1993.

But John Mulvey, executive secretary of the pressure group Save British Science, argues that requiring the funding councils to pay most of the costs of large research equipment is "the wrong way round". Mulvey claims that "it is the OST and the research councils which should be putting in this money".

Powell raises a separate criticism, that the government may be being unrealistic in expecting private sources to come up in all cases with the necessary additional funding.

The mixed reactions to the equipment scheme reflect broader ambivalence towards the government budget allocation to science announced last December, the detailed distribution of which was revealed last week. Taylor himself described the decision to raise the science vote by £30 million, to a total of £1,312 million, as "a very good settlement in the circumstances".

Such views were reflected in statements from several research councils, welcoming their allocations. The chief executive of the Economic and Social Research Council, Ronald Amann, for example, described his council's total budget of £63 million as "a very fair one" in what was "a very tight spending round."

In contrast, however, the Medical Research Council, whose budget will fall by 1.5 per cent in real terms next year, declined to offer the OST a pat on the back for its efforts. In a statement, Sir David Plaistow, the council's chairman, emphasized that the cut "will further erode our ability to support long-term internationally competitive British research".

David Dickson

Britain plans to ask for further cuts in CERN running costs

London. Having successfully led a move last year to secure reductions in the operating costs of the European Space Agency (see *Nature* 377, 687; 1995), the British government is now turning back to a more familiar protagonist, the European Laboratory for Particle Physics (CERN) in Geneva, Switzerland.

Last week, in announcing how the science budget for the next financial year is to be divided between the six research councils (see left), the Office of Science and Technology pointed out that the whole of the science vote will suffer as a result of a dramatic drop in the value of the pound against the Swiss franc — in which the CERN subscription is paid.

The OST has had to earmark an extra £18 million above a baseline figure agreed in 1994 to protect the Particle Physics and Astronomy Research Council (PPARC), from whose budget the subscription comes, against the impact of the shifting exchange rate. "We cannot afford increases of this magnitude," says the OST.

The sentiment is echoed by Ken Pounds, chief executive of PPARC, who said in a statement that he supported the government's view that the rate of increase in the UK contribution to CERN had become "unaffordable".

In the light of the concern, government officials say that they intend to pursue discussions with CERN officials to see whether agreement can be reached on reducing the laboratory's operating costs. British attention is focused in particular on the high salaries enjoyed by the full-time staff of the laboratory. Officials say they accept that the cost of living is high in Switzerland, but point out that many CERN employees live in neighbouring France, where costs are much cheaper.

Last December, Britain joined the German and Italian delegations to the CERN Council in voting against adoption of next year's budget, partly in protest against a new salary award to CERN staff (see *Nature* 378, 758; 1995). Although failing to secure the numerical majority needed to block the budget, the move was seen as a signal of greater pressure to come.

Taylor is convinced that "there are further efficiency savings which could be made at CERN", while Sir John Cadogan, the director general of research councils, says that last December's vote was "a very good sign" that Britain was unlikely to be alone in seeking a new round of savings, particularly given the rate at which the Swiss franc has appreciated in value against other European currencies.

Budget politics jeopardizes US drugs credit

Washington. A popular tax credit that encourages US pharmaceutical companies to produce drugs for rare diseases may soon fall victim to the budget politics that are now embroiling Washington.

The 'orphan drug tax credit' — so called because patients with rare diseases say that in the past they were 'orphaned' by researchers and drug manufacturers — expired at the end of 1994. Its beneficiaries in industry would therefore first feel the bite of its disappearance this April, when their 1995 taxes are due.

Republicans in Congress have included a retroactive extension of the credit, covering the two years 1995 and 1996, in their proposal to balance the budget over seven years. But they are at loggerheads with President Bill Clinton over how the budget should be balanced. Many predict that no agreement will be reached, and if the Republican proposal dies then the tax credit will die with it.

The credit allows companies that develop drugs for rare disorders to deduct half of the costs of clinical testing of the drugs from their tax bill. The US Treasury says the credit cost \$17.8 million in 1992, the most recent year for which figures are available,

and Congress's Joint Committee on Taxation estimates that the proposed two-year extension would cost \$40 million.

The extension would also extend the scope of the current law to allow companies to claim unused credits backward for three years, and forward for 15 years; at present, credits must be applied to the tax year in which costs were incurred. Those likely to gain significantly from such a change are the small biotechnology companies that now carry out about half of orphan drug research; they often do not pay enough tax early in drug development to benefit from the current credit arrangements.

The law defines "rare disorders" as those affecting fewer than 200,000 Americans. Most are relatively rare forms of cancer or genetic diseases, such as Huntington's disease and amyotrophic lateral sclerosis (known as Lou Gehrig's disease or motor neuron disease).

The tax credit was first enacted as part of the 1983 Orphan Drug Act, a package of financial incentives aimed at enticing drug manufacturers into the rare disease market. Drugs that are designated as eligible under the act by the Food and Drug Administration include Amgen's antianemic drug

Epogen and Genzyme Corporation's Ceredase, for Gaucher's disease.

The tax credit has drawn a rare combination of support from both patient advocates and the drug industry. Ironically, both the White House and congressional Republicans also support it. "It's one of the few things that Democrats and Republicans have been able to agree on," observes Lisa Raines, vice president for government relations at Genzyme.

But advocates of the credit are quick to point out that while Clinton endorsed an extension of the credit in his 1996 budget request, and sent a Treasury Department official to the Congress to testify in favour of making it permanent, he did not designate funding for it, leaving that job to Republicans in the Congress.

The Republicans funded the extension in the massive Balanced Budget Act of 1995, which they passed in November and which also contains the new language allowing the credit to be applied forward and backward in time. But Clinton vetoed the entire budget package in early December, objecting, among other things, to its massive projected spending on Medicare and Medicaid, the health programmes for the elderly, disabled and poor. That led to the current impasse, of which the tax credit is a hostage.

Despite the strong political backing for the credit, debt-minded Washington also has its objectors among fiscal conservatives. "This is a tax credit and tax credits are bad economic policy," says Dean Stansel, a fiscal policy analyst at the free-market Cato Institute.

Nonetheless, the provision has found abundant friends among Republicans in the Congress, who see it as a cheap yet effective means of stimulating private investment in medical research while winning friends among both patients and the pharmaceutical industry.

Its broad support may not save the credit from going the way of the balanced budget negotiations. But some observers remain optimistic that the tax credit will still survive. "It will be passed because the [drug] industry wants it, and the industry is very powerful," says Abbey Meyers, the president of the National Organization for Rare Disorders, predicting the passage of separate legislation to extend the credit if the budget negotiations fail.

In Europe, there have been few financial incentives agreed by governments to encourage the development of drugs for rare diseases, but the European Commission has indicated its intention to put forward a directive on orphan drugs. The commission established a task force last year to consult researchers and pharmaceutical companies on the need for new vaccines for a range of diseases.

Meredith Wadman

International papers register decline

Washington. After a decade of continuous growth, international collaboration on published scientific research papers appears to have peaked in 1992-93 and has since started to decline, according to figures released last week by the Institute for Scientific Information (ISI) in Philadelphia.

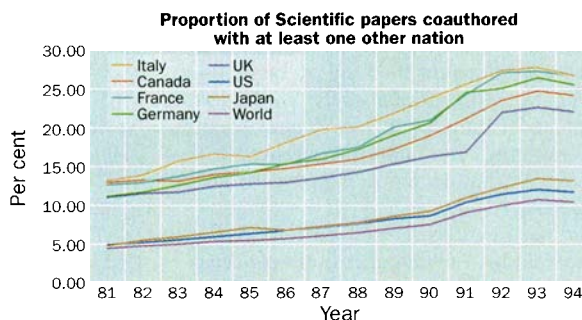
ISI researchers have measured the percentage of papers published in all scientific journals that had at least one co-author working in a different country from the lead author. Their analysis tracks about 10 million papers published between 1981 and 1994. It shows a consistent pattern in all leading industrial nations, with the percentage of papers with international authors peaking in either 1992 or 1993, and falling in 1994.

In countries where international collaboration is more prevalent — European countries and Canada — the level of international collaboration peaked at around a quarter of all papers published. Japan and the United States peaked at half of that level. In the United States, for example, just over 12 per cent of the several hundred thousand papers tracked by ISI had

at least one foreign author; in the following year, that figure fell to 11.7 per cent.

The world average dipped from 10.7 per cent to 10.4 per cent between 1993 and 1994, ending 12 years of uninterrupted growth. Papers with lead authors from smaller countries with high levels of international involvement, including Switzerland and Mexico, echoed the general trend.

Chris King, editor of the ISI newsletter *ScienceWatch*, which published the data, declines to speculate on the cause of the reversal. But the change may reinforce a suggestion by officials of the international division of the National Science Foundation (see *Nature* 379, 3; 1996) that pressure on scientists who wish to retain their grants is discouraging some from engaging in international collaboration. Colin Macilwain



Environmental lobby splits on US tuna bills

Santa Cruz, California. US environmentalist groups are divided over two Senate bills designed to soften the current prohibition on the import of tuna caught using a controversial method that at one point led to the death of 350,000 dolphins a year. At issue is whether it is justified to save one species, albeit one widely embraced by the public, at the risk of endangering other species with less passionate advocates.

One bill, supported by groups including Greenpeace, the World Wildlife Federation and the Environmental Defense Fund, would relax the restrictions on tuna caught using purse-seine methods, providing the methods are modified to result in fewer dolphin deaths. This bill has been introduced by Ted Stevens (Republican, Alaska) and John Breaux (Democrat, Louisiana).

The second bill, sponsored by Barbara Boxer (Democrat, California) and Joseph Biden Jr (Democrat, Delaware), would

change the rules to allow a country to sell its tuna to the United States provided the fish were not caught using any type of purse-seine method. Since 1994 the United States has banned all tuna imports from a country if any of its boats use purse-seine nets.

This bill is supported by organizations that include the Sierra Club, the Humane Society of the United States and the Earth Island Institute, which vociferously object to the Stevens-Breaux bill on the grounds that even the modified purse-seine methods cause discomfort to dolphins.

Pressure to change the current legislation, one of the most popular environmental laws ever passed, comes both from Latin American countries whose tuna is covered by the embargo, and from officials who are responsible for administering the General Agreement on Tariffs and Trade (GATT), on the grounds that this agreement is violated by the American ban.

Fishermen use purse-seine methods to take advantage of an unexplained phenomenon in which mature tuna swim beneath schools of dolphins. This happens in particular in the eastern tropical Pacific (ETP) fisheries, which spread from California to Hawaii and south to Chile.

Fishermen hunt for tuna by looking for dolphins near the surface. A likely catch is then surrounded, using motor boats, and a large fishing boat pulls in a net containing both dolphins and tuna. The tuna are kept, but the dolphins are killed and dumped overboard. Other dolphins that get caught in the net may also drown in the process.

The US embargo on purse-seine tuna has forced many fishing fleets to leave the ETP



Tangled up: dolphins are not the only ones to be caught up in a web of confusion.

South Africa sets out science options

Cape Town. The South African government last week published a long-awaited consultation paper on science and technology which acknowledges that a crisis is confronting the research sector. It suggests various ways in which the country's research administration might be made more effective — but still leaves open the question of which solutions should eventually be adopted.

The green paper was launched in Pretoria by the Minister of Arts, Culture, Science and Technology, Ben Ngubane, who said that it would form the basis of a new science and technology system. Scientists and other interested parties have been invited to comment by the end of February.

Spending on research and development (R&D) in South Africa has declined from a high point of 1.04 per cent of gross domestic product (GDP) in 1987 to 0.68 per cent last year. This fall largely reflects a decline in military expenditure, without comparable funds being reinvested in the civil sector.

The green paper emphasizes that the country's emerging democracy needs to be founded on economic growth. Ngubane has promised to champion the cause of R&D within the government of national unity, despite its many other short-term priorities.

Various options for the government funding of R&D are considered. At present, for example, funding is allocated annually; but the report suggests that it might be done every two or even four years, a proposal that was put forward by Bernie Fanaroff, the senior civil servant who is responsible for the government's reconstruction and development programme (see *Nature* 374, 665; 1995).

The paper also considers how research

funding should be coordinated. The funding of the seven research councils whose budgets make up the science vote is at present coordinated by the department. But the funds are awarded to them through five different ministries, after a cabinet committee has decided on the final allocation of funds between the councils.

The green paper suggests, too, ways of separating out responsibility among the councils for the agency function of awarding research grants and carrying out in-house research. Three of the councils at present carry out both functions, whereas the other four are primarily concerned with in-house research. But the paper does not address the question of whether a clear demarcation of responsibilities for the two categories of funds would lead to a higher proportion being allocated to the academic sector, which has in the past received a relatively low proportion of science funding by international standards (see *Nature* 356, 9; 1992).

In addition, the paper raises the possibility of increasing the scope of the R834-million (US\$230-million) science vote to include the research budgets of the Defence Ministry (R550 million) and the Atomic Energy Corporation (R120 million). Such a move would allow the funds to be reallocated in line with national goals. There is widespread feeling both within the scientific community and within government that these allocations are a hangover from the previous regime.

The green paper will be followed later this year by a white paper outlining the government's position on science and technology, including a clearly defined role for the ministry.

Michael Cherry

for other parts of the ocean. Tinned tuna sold in the United States with the label 'dolphin safe' comes either from free-swimming schools or from fish that gather underneath floating objects, known as 'log sets'.

But many scientists point out that both of these fishing methods themselves result in the deaths of other species, including some that are more endangered than dolphins. The 'by-catch' from school and log set fishing includes sea turtles and sharks, while juvenile tuna can also be killed.

A fishing method known as 'backing down' has recently been developed in which purse-seine nets are tilted down at one end to let the dolphins escape, reducing mortality to fewer than 4,000 a year.

The Stevens-Breaux bill would allow this method and permit tuna to be labelled 'dolphin safe' as long as an observer on each fishing boat saw no dolphins die. But Naomi Rose of the Humane Society of the United States says many dolphins are injured or killed without being seen from the boats.

Niaz Dorry of Greenpeace says the 'dolphin safe' label is a misnomer, as only 20 per cent of the world's tuna are caught in the ETP, and dolphins are still killed when fishermen pursue the other 80 per cent. Reducing by-catch would protect diminishing food fish resources, she says.

Joel N. Shurkin

Bioethics panels may be threat to public debate on research

Paris. The proliferation of bioethics committees risks allowing small groups of individuals to hijack the debate over ethical issues raised by progress in research in biology and medicine, according to Philippe Lazar, the director general of the French biomedical agency, INSERM.

In a book just published in Paris, *L'Éthique biomédicale en question**, Lazar complains that the French national consultative committee on bioethics has changed from a forum for informed public debate to a source of "ready-to-use" solutions to a wide range of ethical problems, often strongly influencing the regulation of new techniques.

Such committees, he says, are inclined to seek consensus on ethical issues, but a lack of consensus is the "essence" of genuine ethical debate in society; a desire for consensus can allow a few individuals to "confiscate" a debate before it has been fully explored. Lazar goes on to question whether France's comprehensive bioethics legislation, finally passed last year, sufficiently respects the diversity of opinions on bioethical issues. Under the heading "A forced consensus?", he warns that Parliament's approval of such legislation may have led the public to mistakenly believe that the problems of bioethics have been solved.

"Nothing could be less certain," says Lazar, arguing that legislation cannot, for example, reconcile the views of a religious individual convinced that human life begins at fertilization, and a hardened atheist who believes in the progressive 'humanization' of the fetus during gestation. A secular democracy needs to accept such differing beliefs, so should refrain from legislating on bioethics, concludes Lazar.

Indeed, Lazar questions whether biomedical ethics in France would be very different if the new legislation had never been introduced. He expresses confidence that society's collective "ideological apparatus" effectively prevents major abuses of new medical technologies. The demand for bioethics legislation, he argues, is symptomatic of society's fear of change.

Mankind's confidence, says Lazar, is now so low that we cannot accept that society is able to make the best use of new research results. This is reflected by an over-emphasis in bioethical debates on the potential abuses of scientific progress, rather than on the new possibilities it offers. "Unconsciously or not", he argues, such emphasis reinforces opposition to scientific progress.

Declan Butler

*Philippe Lazar, *L'Éthique biomédicale en question*, eds Liana Levi, Paris, 1996.



Under wraps: Eden Project hopes to raise funding from Britain's Millennium Commission.

'Clay-pit to paradise' scheme

London. A £106-million (\$160-million) project planned to create a series of massive covered eco-systems within a disused china clay pit in Cornwall is due to be unveiled in London this week, in preparation for a bid for funding from the national Millennium Commission.

The scheme is the brain-child of Tim Smit, and is based on his recent experience in turning a rediscovered Victorian garden, previously used to grow a wide variety of fruits and other plants obtained from all over the world and now known as the "Lost Gardens of Heligan", into one of Cornwall's leading tourist attractions, drawing over 200,000 visitors last year.

But as well as providing a broad educational experience, the scheme — known as the Eden Project — also aims to provide a research environment for scientists keen to study a range of phenomena, from the behaviour of large groups of plants, to the possibility of making commercially valuable products out of Third World crops.

"Initially we will have our own research facilities, but we are also want to become an international institution that outside researchers can use to develop their own research projects," says Smit, pointing out that the size of the scheme alone — the total covered area will be 8 hectares — and its resultant ability to carry out studies of large plant populations, make it significantly different from Biosphere 2 in the United States, whose total covered area is only 66,000 square metres.

Initially four key climatological regions will each be encapsulated in separate but connected 'biomes', namely rain forest, semi-desert, sub-tropics and Mediterranean, on the basis that these produce "not only greater biodiversity, but also the widest range of plants used by man". Visitors will pass through the biomes along a series of walkways, at one point descending from the canopy of a rainforest (see above), at another, crossing a wide stretch of desert.

Smit hopes to obtain funds for each of the biomes from foreign countries — a condition of obtaining money from the Millennium Commission, whose own funds come from the National Lottery, is that grants are matched by outside contributions — and that the research it will be able to house "could provide solutions to many of the issues surrounding global sustainable development". Indeed, any profits from the project will be used to fund research programmes explicitly in line with Agenda 21, the action plan agreed at the Earth Summit held in Rio de Janeiro in 1992.

The Eden Project already boasts an impressive bank of supporters. One key scientific adviser is Sir Ralph Riley, a former secretary of the Agricultural and Food Research Council who remains active in agricultural development projects across the world; Ghilleen Prance, the director of the Royal Botanic Gardens at Kew, and another potential scientific adviser, describes it as "a very exciting project".

The architecture of the scheme has also been planned on a grand scale. The chief architects are Nicholas Grimshaw and Partners, perhaps best known for their award-winning design for the new international terminal at Waterloo Station in London which — like the planned Eden Project — uses broad spans of toughened glass to provide a combination of light and space.

The imaginative scope of the project, including the fact that it will make use of land which is currently a derelict eye-sore, is expected to attract the attention of the Millennium Commissioners, who have been keen to emphasize their interest in innovative projects which can at the same time prove their social value.

But Smit also claims that the criterion of the success of the planned project "will not be the magnificence of its building or gardens", but its ability to act as a symbol of "mankind's shift from exploitation to conservation". □

Astronomers announce discovery of distant massive planets

San Antonio, Texas. Two US astronomers last week announced the discovery of the signature of two massive planets orbiting the nearby stars 70 Virginis (70 Vir) and 47 Ursa Majoris. According to the astronomers, the planets are more massive than either Jupiter or the planet discovered recently near 51 Pegasus (*Nature* 378, 355–359; 1995), and lie further from their stars than the latter.

The planets, reported by Geoffrey Marcy and Paul Butler, have not been observed directly, but their estimated masses suggest that they are gas-giant planets, similar to Jupiter. The stars are both similar to the Sun, and both lie about 10 parsecs away. The mean temperature of one planet, that around the star 70 Vir, has been estimated to be about 350 K, but its orbit is extremely eccentric, so and the temperature will vary substantially over its 110-day 'year'.

Indeed, the planet is so eccentric that some astronomers even question whether it should be called a planet, as current understanding of the formation of such bodies suggests that they should lie in low-eccentricity orbits. The presence of a planet with a mass comparable to Jupiter only 5 million miles from a star — as is that discovered last year — combined with the newly revealed eccentric orbit of that close to 70 Vir suggest that much remains to be learned about the formation of solar systems.

Leslie Sage

Head for Japanese space institute

Tokyo. Atsuhiko Nishida, deputy director-general of the Institute of Space and Astronautical Science (ISAS) in Kanagawa, Japan, has been appointed as the institute's next director-general. He succeeds Ryojiro Akiba.

Nishida, who will turn 60 in March, has been a professor at ISAS since 1981. He has held the post of adjunct professor at the University of Tokyo during the same period. A solar-terrestrial physicist,

Nishida has been involved in the GEOTAIL project, a joint ISAS programme with the US National Aeronautics and Space Administration, to investigate the Earth's magnetic tail. Nishida has also been vice president of the International Council of Scientific Unions' Committee on Space Research (COSPAR) since 1994. □

Germany elects woman president

Munich. Dagmar Schipanski, a member of Chancellor Helmut Kohl's technology council, has been elected to head Germany's influential science council, the Wissenschaftsrat. Schipanski, an electronic engineer from Berlin, will be the first woman president of any German research organization.

An east German who spoke out against the French atomic tests, Schipanski may resurrect the political profile of the Wissenschaftsrat. Her predecessor, Karl-Heinz Hoffmann, had avoided controversy during his two-year term of office, breaking a tradition established by his predecessors, Dieter Simon and Gerhard Neuweiler, who had energetically campaigned for extensive reforms in universities and research institutes. □

Tighter grip on Russian funding

Moscow. Victor Chernomyrdin, the prime minister of Russia, has decided to take personal control of science funding, according to a report from the Interfax news agency. The decision was announced at a meeting held last month to discuss ways of implementing an earlier parliamentary resolution on state support for research and science.

Boris Saltykov, the minister for science and technology policy, told the meeting that science funding for 1995 had been incomplete and erratic, and that only 80 per cent of the agreed science budget had been made available. Academic and university research received 75 per cent of its allotted share, and even top priority research programmes had only received 62.5 per cent, said Saltykov.

Chernomyrdin demanded that Russia's finance minister should

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The enemy of science is within

SIR — John Maddox's puzzlement (*Nature* 378, 435–437; 1995) at the generalized public distrust of science should be pursued. If Democritus's assertion (a cornerstone of Baconian science), that all things occur according to natural law or by chance were to permeate the public understanding, some interesting things might happen. For example, the trivial television series *Star Trek* would fail to appeal to a public forearmed with a belief in the tidiness of thermodynamics. The myth of miracles and the almost universal doctrine of supernatural intervention would be recognized as intoxicating folklore, and limiting population in the interests of the environment might become possible. The resurgence of vitalism in the 'natural' food industry, where much of society believes that a radish nourished on manure is preferable to one treated to ammonium nitrate, would also be due for an overhaul.

It seems to me that the public distrust of science is mostly due to the abject failure of scientists to make the case that we live in a material Universe. Scientists have engaged in a peculiar apostasy in dividing their beliefs of science on the one hand and succumbing to ethnic and cultural fantasies or, to paraphrase some popular wisdom, (1) if science has an enemy, it is us, and (2) if we are not part of the solution, we are the problem.

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SIR — I am in sympathy with Maddox's comments about "the prevalent distrust of science". To me the centrepiece of the article is the phrase that "to represent the nature of human beings by a description of their genes undermines their dignity as human beings" and that "research in human genetics" is therefore to be opposed.

Despite its brevity, this statement glows with misapprehensions and unreflected prejudices, and it is this cocktail of stupidity that is the actual poison. To evoke as an antidote, as it were, the benefits that people enjoy as the results of scientific endeavour is useless labour, because these benefits constitute a category different from, and so irrelevant to, the categories of misapprehension and prejudice.

Hope, if there is any, rests with enlightenment alone. But does Maddox really believe that church (in fact, and so to speak, his Commentary's last word) is the place where the light will eventually shine? To me it appears to be the place where people are indoctrinated with pre-

conceptions about "the nature of human beings" that are ultimately baseless and consequently do not help us at all when it comes to solving the problems of our time.

Helmut Grünewald

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SIR — Maddox provides an example of why distrust of science persists. If the contribution of science were as one-sided and positive as he suggests, such distrust might be hard to understand. However, given that science is done by humans within a cultural and historical context and usually reflects the values of the culture, the argument that only the positive results count is naive. A glance at the history book should provide sobering detail of science in the service of death and destruction. The rosy misrepresentations Maddox provides are worthy of our distrust.

Stephen Keast

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SIR — Maddox makes several suggestions as to how the worrying trend towards public distrust of science can be reversed. He rightly stressed the importance of scientists not overstating the implications and value of their work and of the need to make the public more aware of how the scientific process works. He also recognized how science can appear to be a threat to those with deeply held religious beliefs, and challenged those of us who are both scientists and 'religious' to play our part in showing the fallacy of such fears.

The point is well taken, and there are many of us who find opportunities within the church and other religious communities to do just this. But there is an equal responsibility for scientists to avoid making unnecessary and intolerant attacks on religious belief. If the impression is given by some scientists that religious belief must conflict with the scientific view of the world, it should be no surprise that many in the population, for whom religious belief is a real and vital experience, distrust science and scientists. Those who seek to preach a 'gospel of science' that opposes religious belief are more likely to accentuate any distrust of science in the majority of the population than they are to convert others to atheism.

Andrew P. Halestrap

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Confidentiality of referees

SIR — In his valediction, John Maddox (*Nature* 378, 521; 1995) raises "the problem of the implied confidentiality [of referees]". The solution to this problem is clear: eliminate it. Since its inception 15 years ago, *Cell Calcium* has requested referees to sign their reports. I can recall only a handful of occasions when a referee has declined to act because of this stipulation.

While *Cell Calcium* is clearly not *Nature*, it is a highly regarded journal. (At the risk of incurring Maddox's wrath by mentioning "yardsticks of attainment", let me say that it has an impact factor of around 5). As co-editor (with Toni Scarpa) of this journal, it is my subjective impression that a requirement to sign a report, while reducing the invective, in no way lowers the scientific rigour of referees who, unable to hide behind unsubstantiated generalizations, are forced to focus on facts. Isn't that what science is all about? I cannot believe that science (or indeed almost anything else) is best served by confidentiality. I therefore find it rather depressing that, after 30 years at the helm of a leading science journal, Maddox still feels that it is important (while admitting that in practice it doesn't exist).

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Family likeness

SIR — Nicholas Christenfeld and Emily Hill's fascinating analysis of the similarity between young children and their parents showed that babies are recognizably similar to their fathers and apparently less similar to their mothers (*Nature* 378, 669; 1995). They argue that evolution would select babies who look like their father to reassure the father that the baby is his. That cannot be correct. How would pre-industrial fathers know what *they* looked like? They had no mirrors.

It may be that this signalling system is aimed at the mother, or at other men or women, to tell them that this baby belongs to this man. Or it may be that, in underlying facial morphology as in other matters, men remain more infantile than women.

William Bains

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Biological anthropology

SIR — Your report of Roger Bannister's views on the biological basis of exceptional track athletes (*Nature* 377, 183–184; 1995) characterized biological anthropology as being “under a cloud for its habit of using measurable skeletal indices as proxies for less tangible attributes” such as cranial capacity as a measure of intelligence. It was suggested that this should be replaced by a modern focus centred in the proposed Human Genome Diversity Project (HGDP).

No life science can ignore modern genetics, but the HGDP would at most be a supplement to rather than a replacement for the diverse work of biological anthropologists, which includes palaeontology, growth and development, and behaviour studies, to name but a few. Work in these areas is of contemporary quality, and contributes to our understanding of *normal* human variation and its origins, a subject too often omitted in biomedical research.

Physical anthropology has not used such indices as cranial capacity as proxies for human intelligence for decades. Indeed, textbooks and instructors in anthropology go out of their way to stress the fallacies and dangers in such usage.

This is an era of rapid discovery of important and wide-ranging new knowledge in genetics. The resulting intense competitiveness can lead to hubris and excessive claims about the power of genes to determine almost any human trait, including socially relevant ones. Unfortunately, this hubris is matched by a class of comparably unrestrained professional critics who exploit the widespread public misunderstanding and fears of genetics to suggest that the study of human genetic variation is almost necessarily sinister. The HGDP has never claimed that the type of genetic data it would collect would be relevant to studies of variation in traits such as athletic performance or intelligence, and certainly not as pertains to ‘racial’ stereotypes.

On the contrary, properly done studies of human variation, genetic or otherwise, can contribute to a more inclusive, less classificatory understanding of our nature as a species. On the basis of what we already know, one consequence of such studies of genetic diversity will not be to usher in a new era of genetic determinism but to enhance our recognition that human biological phenotypes — such as running ability — are not categorically distributed among different ‘races’. Nutrition, habituation (training), growth and development, and cultural aspects of human beings make material contributions to all such traits. This is the routine subject matter of anthropology.

Africans who have been competing successfully in international track and field

should be deservedly proud of their efforts. Were this just in their genes, we could simply award Africans a permanent Gold Medal and abandon future Olympic races. That would diminish their achievement, making it seem automatic, a loss to the whole purpose of sport. And it would be bad genetics, as any good biological anthropologist would tell you.

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Write to reply

SIR — In John Maddox's parting leading article (*Nature* 378, 521–533; 1995), he repeats a theme often sounded before: that scientists are poor writers (or at least that scientific papers are poorly written), and speculates on the reasons. One reason is that we are forced by our colleagues to write obscurely. This is never mentioned in the endless discussions about scientific writing, but it is true nonetheless.

For instance, Strunk and White advise writers, “Use the active voice”. Robert Day adds, “Do not be afraid to name the agent of the action in a sentence, even when it is ‘I’ or ‘we’”. Following their advice provoked this response from a referee: “Most of my comments concern writing style. My biggest preoccupation with style is that the paper is written in the first person. This should be avoided whenever possible.”

Here is some more advice given to authors: “Every scientist should avoid jargon” (Day). “Shortness is a merit in words” (Fowler). “Avoid fancy words” (Strunk and White). But scientists who have followed it know that their colleagues don't agree. The word “get” in their manuscripts is crossed out and replaced with “obtain” (occasionally with the comment “colloquial” in the margin). “Use” becomes utilize, “method” becomes (usually incorrectly) “methodology”, and so on.

The worst sin is liveliness of style. Many scientists are earthy in speech but can't distinguish dignity from pomposity in prose. Lively writing will usually provoke criticism. If you attempt to include an actual JOKE in a scientific paper, you have a major fight on your hands.

Peer pressure can't completely explain why scientific writing has become so dark and dull. One has still to explain how we scientists picked up this dismal habit. But peer pressure can explain why the habit persists. A young scientist who tries to write

well gets stamped on. To avoid the risk of inflaming a referee and having a paper rejected, he desists. In time, the belief that “get” is a colloquialism so grows on him that he automatically crosses it out whenever he sees it.

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Joule ratings miss the mark

SIR — Even without possible fraud (see *Nature* 377, 281; 1995), the project review procedure of the European Commission (EC)'s Joule programme lacks scientific integrity. Each project proposal seems to be evaluated by some five referees, each giving the project a rating from A to N. The ratings are then averaged, and applicants may learn their average rating from their national focal points. However, in contrast to other EC programmes, which transmit both rating and some ten lines of reasons for the verdict to each proposer, the Joule programme provides no feedback to applicants.

The procedure used in the Joule programme inevitably leads to the most innovative project proposals being rated C or below because of differences in the outlooks and preferences of the reviewers. Because there is money only for the A-rated projects and perhaps a few B-rated ones, some of the more challenging projects are lost, and it is the least controversial that seem to be supported. The list of funded projects seems to confirm this, as does my personal experience of always getting money for the project proposals about which I was least enthusiastic. It may also explain the incorrect impression of Ezio Andreta, who is in charge of the section of the research commission concerned with non-nuclear energy, that the number of worthy projects is diminishing. (He probably does not see the proposals turned down by the scientific review teams.)

My conjecture may not be valid in more narrow areas of science, but the commission's programme areas are always broad and interdisciplinary. Different schools and skills will therefore rate a particular project differently, and, assuming that the width of the fields is indeed reflected in the choice of referees, the inevitable result will be the loss of many innovative project ideas, which will never achieve a consensus A-rating.

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Ethics of genomic patenting

SIR — George Poste, in setting out Smith-Kline Beecham's "case for genomic patenting" (*Nature* 378, 534–536; 1995), has too readily implied that it is accepted by the Nuffield Council on Bioethics. In its report, *Human Tissue: Ethical and Legal Issues*, the council made three relevant points (pp 135–136):

"We recognise that inventions derived from human tissue are open to patenting. Over two hundred patent applications have been published where the criteria for patentability have been met. We accept this position as a matter of fact.

"There is at present a major controversy about patenting in the area of human genes. The law, as it stands, discriminates between discoveries and inventions. Fundamental to the application of the notion of invention in this area is that some technical intervention should have taken place that justifies the granting of an intellectual property right. We note that questions of fact arise in each case on whether patent applications meet the existing legal criteria.

"We attach great importance to the fuller consideration and review of the process by which ethical issues are taken account of in relation to the question of patenting inventions derived from human tissue. We recommend that the Government joins with other member states of the European Patent Convention (EPC) in adopting a protocol to the EPC which would set out in some detail the criteria to be used by national courts when applying the immorality exclusion to patents in the area of human and animal tissue."

The council's 'endorsement' of patenting was directed primarily at 'inventions derived from human tissue', and in particular at applications to patent cell-lines. On the patenting of human genes, the council reserved its verdict, while stressing the distinction drawn in law between discoveries and inventions, which causes difficulties for patent offices in this area.

On my personal reading of Poste's discussion of SmithKline Beecham's policy, and Merck's policy on genomic patents, the latter's ethical position has much to be said for it, not least that it maintains the distinction between discovery and invention.

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Austrian science

SIR — Research in biochemical sciences in Austria was recently evaluated by a dozen colleagues from different countries selected by the European Molecular Biology Organisation (EMBO) (see *Nature* 377, 468; 1995). These experts took on a formidable task and produced an objective and

balanced review.

But the *Nature* article does not truly reflect the tenor of the experts' report. The introduction to the report says that "[i]n several areas of biomolecular research very good and excellent work is being carried out", yet "the greater part of Austrian biomolecular research cannot be classified as being internationally leading or competitive" and "a relatively large number of groups have to be classified as poor in their scientific output".

Unfortunately, the *Nature* article dwells mainly on the most negative statements of the introduction and fails to consider the analysis of the individual research groups which reveals a more positive situation. Thus, of the 79 groups evaluated (from 8 universities and a few other institutions), 54 were rated as good or very good, the rest as average or poor. We fully agree that each group in the lower two categories is one too many, but we believe that biomedical research in Austria is not at the level you depict. Moreover, after the evaluation, Professor G. Czapski (Jerusalem) carried out a detailed statistical analysis of Austrian publications in the biochemical sciences for the Austrian Biochemical Society. In this analysis Austria ranked fifth and seventh (among 58 countries) with respect to the proportion of top papers in biochemistry/biology and molecular biology/genetics respectively.

A fair and objective comparison of biochemical research in Austria and its European neighbours is currently not possible. Only when other countries have come under the scrutiny of similar impartial committees can a valid comparison be made. The Austrian Biochemical Society agrees with a large part of the assessment of individual groups in the report by the EMBO panel and acknowledges that the number of internationally recognized scientists is too small and the average scientific productivity too low. We face the challenge to increase both and hope that the report will lead to changes in employment and funding policies in Austria that will reward scientific productivity.

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Foreign workers in Europe

SIR — As an American postdoc for the past two years in both France and Germany, I feel compelled to comment on both your figures and their interpretation in your leading article about Germany (*Nature* 378, 755; 1995). There appears to be a disparity between the number of European Commission (EC) Human Capital and Mobility (HCM) fellowships going to Britain, France and Germany. However, of the three, only Germany has significant internal funding sources for foreign postdocs. By contrast, foreigners in France and the United Kingdom are almost completely dependent on the EC Fellowship programme. If one were to study all postdocs in Europe (not just those sponsored by the EC), I am certain Germany would be better represented.

As for the language and bureaucratic barriers to foreign scientists in Germany, my experience after a year each in Germany and France is exactly opposite to what you report. During the time I was employed by the French CNRS, a law (deplored in these pages) required me to conduct all my daily scientific work in French. Few of my colleagues, though able, were willing to speak English at work anyway. It was nearly impossible to conduct any science there without a working command of French. The CNRS paid for French lessons for me, and I enjoyed learning, but it took massive amounts of research time away to do so. Here I need speak almost no German on the job at all, even though my German is already nearly perfect. However one may feel about that, language is not a greater barrier to foreign scientists here than in France. My residence permit took an hour to get in Germany; in France it took 6 months, several lost work days, and repeated humiliation by bureaucrats in the Hotel de Police and the Mairie. My experience was not unique, the price I suppose for an otherwise happy and productive year in France.

And I experienced much more *Ausländerfeindlichkeit* in France than here, often expressed openly by government officials in the course of their duties, and without much reflection. Such a thing is impossible here, now, without a huge public uproar. If the point of your leading article was that foreign stereotypes about Germany do not square with reality, you are clearly right. You imply that Germany is in fact less welcoming to foreign scientists than other European countries: there you are wrong, in my view.

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Melatonin hype hard to swallow

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Melatonin is being touted as the latest cure-all. But in their eagerness to explain to the public the possible health benefits of this natural hormone, the mass media as well as some scientists have misrepresented the scientific data.

AN advertisement at the check-out counter of a large drug store in Chicago runs: "You have seen it on TV and read about it in major national publications. Buy a bottle today and see for yourself why melatonin has everyone talking." Why are they talking? By some accounts, a single melatonin pill will cure or prevent cancer, heart disease, Alzheimer's disease, diabetes, cataracts, AIDS, depression, schizophrenia, sudden infant death syndrome, epilepsy, autism, Parkinson's disease, jet-lag and influenza. It will improve your sex life, reverse the ageing process and help you to sleep better and lose weight. Clearly, this 'natural' compound is no less than the fountain of youth for the ageing baby-boom generation. Such wild claims have been made in the past for other compounds, and will no doubt be made in the future; they are similar to those made by salesmen who rode their covered wagons from town to town in the Wild West of nineteenth-century America selling 'snake oil' as a miracle cure. But the message about this latest miracle cure for all health problems is not being delivered from the side of a covered wagon; rather, it is being advertized and promoted internationally in a series of new books, in newspapers and magazines, on television and radio and, in the United States, in health-food and drug stores across the country.

Biomedical backwater

Melatonin is a hormone that in vertebrates is produced by the pineal gland, a pea-sized organ at the centre of the human brain. Until recently, the mammalian pineal gland was often referred to in medical texts as a vestigial organ, and was believed to have no function at all in humans. The discovery in the early 1950s that the pineal in fact produces a substance secreted into the bloodstream led a few investigators to look for a role for this new hormone and the gland that produces it. But over the next 20 years the biomedical profession as a whole paid little attention to this challenge. In the early 1970s, my own interest in melatonin prompted one eminent endocrinologist to tell me that the pineal gland and melatonin had been the graveyard of many a young scientist. So after languishing in obscurity for so long, how did melatonin rise to almost instantaneous worldwide fame?

The current excitement about melatonin is in fact rooted in many years of scientific investigation not only into the pineal gland and its function but also into daily (circadian) and seasonal rhythms in animals. The daily light-dark cycle regulates the production and release of pineal melatonin into the blood: in all vertebrate species (whether nocturnal or diurnal), melatonin levels are low during the day and high during the night. So the diurnal rhythm in circulating melatonin levels could provide a signal to systems throughout the organism indicating when it is day or night; and by providing a measure of night length and thereby day length, the duration of high melatonin levels could indicate to the organism which season of the year it is. In birds and other lower vertebrates, removal of the pineal gland or continuous delivery of melatonin severely disrupts the expression of 24-hour rhythms in rest and activity or in body temperature^{1,2}. Although in many mammalian species such treatment alters the annual reproductive cycle regulated by day length^{3,4}, it does not cause any major disruption of mammalian circadian rhythmicity⁵. But daily injections of melatonin can set the timing of the free-running rhythm of activity in rats maintained in constant dim red light⁶, an effect that is also found in blind humans⁷.

While melatonin and melatonin-related drugs were being tested for their chronobiological properties, other studies were showing that melatonin could influence several cellular processes that might in turn influence a wide variety of physiological systems, including the sleep and immune systems. These results, together with the finding that melatonin levels markedly decline with age⁸, led to several hypotheses about the role of melatonin in ageing and age-associated diseases, and hence to the present frenzy over melatonin therapy.

So how did a great deal of serious and medically important research turn into the 'melatonin craze'? The short answer is simple: scientists went public, writing books and articles for a general audience and giving interviews on radio and television. This of course is precisely what scientists should be doing in the current climate of 'distrust of science'⁹ and at a time when public and government support of science is diminishing. Indeed, it is

clearly the duty of the many scientists whose careers are heavily dependent on taxpayers' money to inform the public of the possible benefits of their work and the importance of scientific research in general. But in spreading this message, scientists must be careful not to cross the 'truth-in-advertisement line' by exaggerating the significance of a few selected studies to the point where the public receives an unbalanced and potentially dangerous view of the present state of knowledge.

Going public

Two popular books fuelling the melatonin craze are representative of many other recent volumes written by scientists trying to take their message to the public^{10,11}. One clearly crosses the frontier of scientific objectivity, whereas the other shows some regard for the importance of that frontier. Both books raise the hope that a 'magic bullet' has been found for many of the world's biomedical woes. But in *The Melatonin Miracle*, Walter Pierpaoli and William Regelson present many statements of fact about the health benefits of melatonin that on closer inspection often turn out to be merely hypotheses with little supportive data; indeed there are often no data for humans that directly support many of their claims. What is more, evidence contradictory to these hypotheses is invariably ignored.

In a chapter on "Melatonin: For a Strong Heart", for instance, the reader is told that melatonin brings blood cholesterol levels back to normal, lowers high blood pressure and helps to prevent heart attack and stroke. But not one study — controlled or uncontrolled — in humans is cited that tests whether melatonin supplements can do any of these things. Reference is made, though, to a friend of one of the authors who began taking 5 mg of melatonin nightly; when the author met him a few months later, he noted that his friend was stronger and healthier and that his blood lipid and sugar levels had returned to normal. Yet we do not learn if the friend was taking any other medication that may have been responsible for these changes.

Although so far there have been no studies on humans to establish the effects of melatonin on heart disease, the authors conclude that "[m]elatonin is an

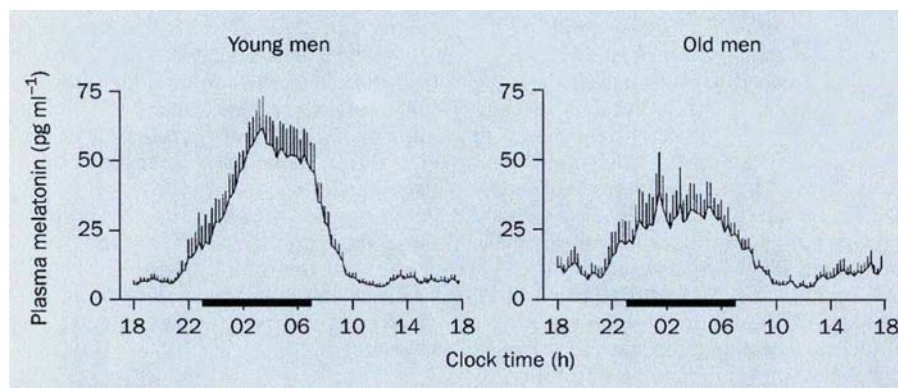
extremely useful tool in helping to prevent heart disease, the number one killer of both men and women" and "[i]n sum, melatonin will help to keep our hearts strong and efficient for our entire lives". And in a chapter on "Melatonin and Sex" the authors claim that "[i]f you have ever had an erotic dream, you can thank your pineal gland" and "if you have ever been sexually aroused, you should be grateful to your pineal gland", although they fail

the potential benefits of taking melatonin. Although some of the statements on the jacket cover of his recent popular book *Melatonin* have crossed the truth-in-advertisement line, Reiter and his co-author, Jo Robinson, repeatedly say that the data are promising and suggestive, but that more work needs to be done to discover whether taking melatonin is beneficial. For example, in saying "Can melatonin help reverse the age-related decline

indication, one can find both positive and negative results (for effects on sleep, for example, see refs 13 and 14). More animal studies obviously need to be done, not only to determine if treatment with melatonin can attenuate or reverse some of the effects of ageing, but also to determine the mechanisms of any such effects. Clinical research studies and large-scale clinical trials are needed to demonstrate whether melatonin supplements can indeed have beneficial effects in the elderly, and to rule out the possibility of toxicity. Alas, few drug companies will be interested in such trials: they are expensive, and, because melatonin cannot be patented, they have little potential payoff. Until patentable analogues of melatonin are used in large-scale clinical trials sponsored by the pharmaceutical industry, unwitting customers of drug and health-food stores will be the test subjects for melatonin, without there being any scientific evaluation of its effectiveness or toxicity.

These are exciting times for research on melatonin and the implications of its use. With the tremendous public interest in its possible benefits, workers in the field now have an ideal opportunity to build on a foundation of research that spans 40 years to establish if the hormone can indeed alleviate or remedy specific human diseases. There has always been, and probably always will be, public enthusiasm for quick snake-oil cures to complex problems, and the scientific community, in taking its message to the general public, must stand with both feet on the 'right' side of the truth-in-advertisement line. Otherwise, both the scientific profession and the public will be losers. □

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Daily plasma melatonin levels in young men (20–27-year-olds) and old men (67–84-year-olds) (mean + s.e.). Despite the decline in maximum nocturnal levels of melatonin with age, there is little direct evidence in humans that melatonin supplements can attenuate or reverse age-related changes in health. Black bar, bedtime. Adapted from ref. 8.

to give any evidence from any studies in humans that would support such claims. In fact, in many animals treatment with melatonin is associated with gonadal atrophy⁴.

On and on it goes. The authors claim with certainty that the pineal gland controls circadian rhythms, including the sleep-wake cycles in humans and in animals, as well as the onset of puberty in humans. But virtually every scientist studying circadian rhythms or puberty would disagree with these statements. The reader is also informed unequivocally that: "The pineal gland is the body's aging clock, the internal timer that controls the aging process. It releases the hormone melatonin, which transmits instructions to other body systems that tell them how and when to age." We are never told how few data there are to support such a sweeping role for the pineal gland or melatonin in the ageing process, or the potential flaws in the experiments cited in support of the claim that the pineal gland is the body's ageing clock. In Pierpaoli's own studies of ageing, the pineal glands of young mice were transplanted into older animals, and vice versa, with corresponding effects on health and longevity. *The Melatonin Miracle* conveniently omits to mention that the strains of mice used in these studies do not produce melatonin¹² or that these "melatonin free" animals show normal 24-hour rhythms and have normal pubertal development.

Russel Reiter, a pioneer in the field of pineal research, is also a firm believer in

immunity? The animal research gives us reason to hope", the authors at least admit that here they are talking about theory rather than fact. Throughout the book the reader is told about the exciting results indicating that melatonin can be important for human health, yet the many uncertainties and unanswered questions are not ignored: "People who take melatonin at this time are facing a lot of unknowns. Even we researchers have questions about how to use it." As Reiter concludes: "The conservative course of action is to refrain from taking melatonin at the present time. In five years, we should know a great deal more about the possible benefits and risks of taking the hormone."

A principal theme in *Melatonin* is that much more research is needed, particularly when one considers one of the most exciting therapeutic uses of melatonin: as an anti-ageing compound. The figure shows the decline with age of the daily rhythm in circulating melatonin. (Similar age-related declines in other hormones, such as growth hormone and testosterone, have also been observed, and many investigators are examining the importance of the decline in a number of different hormones in the ageing process.) This decline is provocative, particularly as melatonin affects several physiological systems that deteriorate with age. But many of the claimed associated benefits of melatonin are based on a handful of small experiments or on individual testimonials, and the data are simply inconclusive: for just about every

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The G-protein nanomachine

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Many of the external signals reaching a cell are transmitted through G proteins, which lie just inside the cell membrane. New crystal structures shed light on how the complex works.

HUNDREDS of cell-surface receptors employ G (for GTP-binding) proteins to initiate intracellular signalling chain reactions which guide a cell's actions. These proteins consist of three subunits, α , β and γ , forming one of nature's most important miniature (nano) machines.

On pages 311 and 369 of this issue^{1,2} and in a December issue of *Cell*³, collaborating protein biochemists and crystallographers from two pairs of laboratories (Sigler-Hamm of Yale University and the University of Illinois, Chicago, and Sprang-Gilman at the University of Texas, Dallas) have put in place one of the remaining pieces of the G-protein puzzle. Their achievement is to have solved the crystal structure of the G-protein heterotrimer ($G_{\alpha\beta\gamma}$) and, particularly, of the mysterious $\beta\gamma$ dimer ($G_{\beta\gamma}$). The whole signalling complex does not act as a set of sequential bimolecular reactions, but rather like a nanomachine consisting of a lever (receptor), switch (G_{α}) and propeller ($G_{\beta\gamma}$).

The cell is surrounded by a lipid moat defending the soluble, organized and energy-requiring intracellular world from the surrounding chaos. But even a unicellular organism must engage the outside world in order to mate with, flee or eat its neighbours. One, and perhaps the most common, evolutionary mechanism for passing notes across the lipid barrier is the G-protein signal-transduction system (Fig. 1).

The genome has devoted roughly a

thousand of its 100,000 or so genes to specific receptors which span the cell membrane in a serpentine fashion, threading themselves seven times across the lipid bilayer; these receptors connect with G proteins just inside the cell membrane. We are least aware of the taste and smell receptors for alien small molecules, estimated to make up the bulk of these proteins. The other receptors (perhaps a few hundred) are specifically bound by intercellular messenger molecules such as acetylcholine, glutamate, γ -aminobutyric acid, adrenaline, dopamine, histamine and opiates; some are even adapted to recognize photons. Vision, smell, taste, neurotransmission and numerous hormonal responses are mediated by these fairly choosy receptors, and much of the physician's tool chest relies on binding and activating or blocking them.

G proteins perch just inside the plasma membrane on lipidic feet (G_{α} subunits may be myristoylated and palmitoylated; G_{γ} of the $G_{\beta\gamma}$ dimer, subunits may be farnesylated or geranylgeranylated). The receptor they serve is not just a passive keyhole; when bound it changes shape and its intracellular domain inserts itself to catalyse the release of GDP from G_{α} 's grasp. Cytoplasmic GTP and GDP levels are high (not limiting) and GTP replaces GDP in the G_{α} cleft, initiating conformational changes in two 'switch' regions of the G_{α} subunit. GTP-bound

G_{α} and $G_{\beta\gamma}$ subunits are set adrift.

A decade ago it was thought that the freed GTP-bound G_{α} subunit was the main player in this drama. Purified G_{α} activated the enzymatic activity of cGMP-phosphodiesterase (the effector in rods and cones of the vertebrate eye), adenylyl cyclase and phospholipase-C β . $G_{\beta\gamma}$ was thought to act merely as a lipid-bound docking site waiting for G_{α} 's return. In 1987, clear evidence was obtained that $G_{\beta\gamma}$ activated its own effector, in this case a K⁺-selective ion channel (I_{KACH} , GIRK1/CIR channel)⁴ responsible for nerve-mediated slowing of heart rate, and it has now been shown that $G_{\beta\gamma}$ directly binds and activates numerous effectors⁵ (Fig. 1). But definitions such as activation may turn out to be arbitrary simplifications, resulting from experiments that usually detect only one consequence of a protein's conformational change. In the case of β ARK (β -adrenergic receptor kinase), $G_{\beta\gamma}$ positions the target protein, the kinase, close to its receptor substrate⁶.

Why employ all this just to generate further signals, so-called second messengers, in the cytoplasm? Receptors have two specific interfaces: one with an external ligand, and a second with an intracellular G-protein heterotrimer. At least 20 G_{α} , five G_{β} and 12 G_{γ} subunits provide significant combinatorial signal-transduction options. The bipartite signal of released G_{α} and $G_{\beta\gamma}$ also doubles the

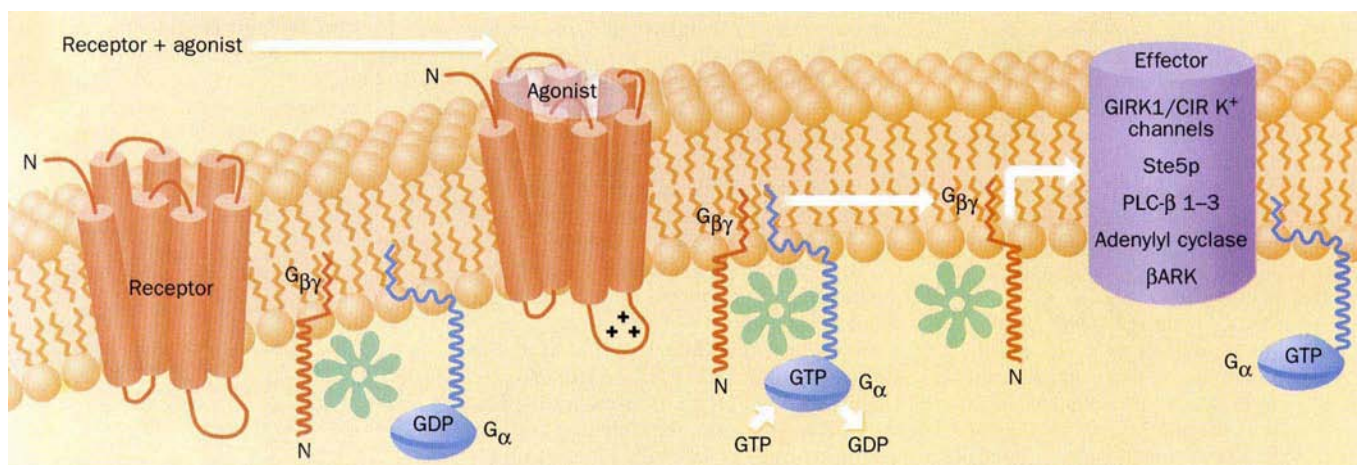


FIG. 1 G-protein-linked receptors catalyse the exchange of GTP for GDP on the $G_{\alpha\beta\gamma}$ heterotrimer. The separated G_{α} and $G_{\beta\gamma}$ dimers activate various enzymes and ion channels. $G_{\beta\gamma}$ is known to regulate the effectors listed, including the I_{KACH} (GIRK1/CIR) channel, a yeast MAP kinase scaffold (Ste5p), phospholipase-C β (PLC- β) types 1-3, adenylyl cyclases and G-pro-

tein-linked receptor kinases (GRKs). Other proposed effectors directly or indirectly regulated include the MAP kinase pathway (perhaps involving Ras, Raf-1, Shc adapter protein, phosphoinositide-3-kinase and various tyrosine kinases), voltage-activated N/P/Q type Ca²⁺ channels, phosducin, plasma membrane Ca²⁺ pumps and phospholipase A₂.

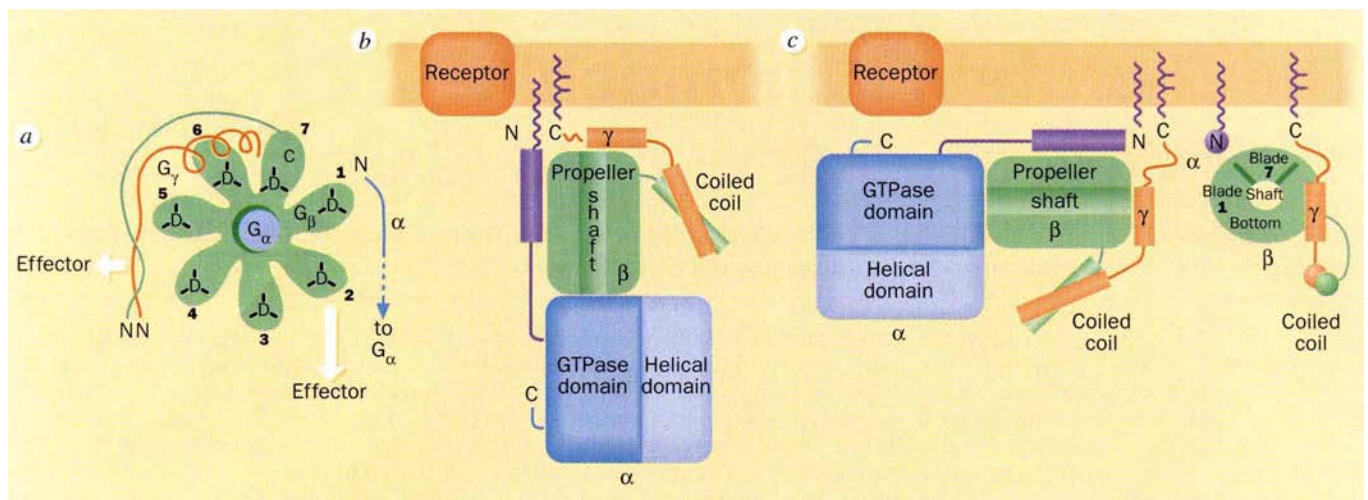


FIG. 2 Functional interfaces revealed by crystallography of the $G_{\alpha\beta\gamma}$ heterotrimer and by mutagenesis. *a*, The $G_{\beta\gamma}$ propeller as seen from the cell membrane looking down the propeller shaft to G_{α} . The outer part of the blades are least conserved. Previous work predicted correctly the $\beta\gamma$ amino-terminal coiled-coil, the importance of the region now known as blade 5 in β 's interaction with γ , and the requirement of the G_{α} amino terminus for interaction with $G_{\beta\gamma}$ ¹². Conserved regions of 40-amino-acid stretches, particularly a conserved aspartic acid (D), are needed for structural integrity. *b*, One potential side-view of the G-protein heterotrimer. The lipidic C terminus of G_{γ} and N terminus of G_{α} anchor $G_{\beta\gamma}$ and G_{α} to the membrane. The variable amino terminus of G_{α} is needed for $G_{\beta\gamma}$ binding. The middle region of G_{γ} establishes G_{γ} - G_{β} interaction specificity. G-protein-linked cationic domains, perhaps amphipathic

α -helices, interact with the G_{α} carboxy terminus to trigger nucleotide exchange. The switch domains of G_{α} face the electronegative bottom of G_{β} , probably partially burying effector interfaces of both G_{α} and $G_{\beta\gamma}$ and protecting the switch domain. Upon GTP binding, the G_{α} switch domain shifts and compacts the G_{α} structure. $G_{\beta\gamma}$ does not alter its conformation when released from G_{α} . The coiled coil on the left and the variable tips of the blades may provide specificity of interaction of $G_{\beta\gamma}$ with receptors and effectors. *c*, Most probable side-view of the heterotrimer. The electroneutral region of the 1/7 propeller blades (see Fig. 4a of ref. 1, page 318) and the G_{α} and G_{γ} lipidic modifications anchor the complex to the membrane, rotating the propeller 90° with respect to part *b*. The G_{α} switch region is theoretically now more accessible to the receptor. At right, a view towards the propeller face of the complex.

number of classes of simultaneous effector endpoints. G_{α} subtypes' varying hydrolysis rates provide a range of kinetics and an accessible control mechanism. In the end, the nanomachine leads to tremendous potential amplification in numbers of endpoints, control mechanisms or turned-on effector molecules. A few photons impinging on the retina reach our consciousness as a result of the amplification, 10,000 times or more, of signal molecules generated by this system.

Years of painstaking protein biochemistry and mutagenesis provide some insight into the workings of the signal-transduction complex. But in one fell swoop, G-protein crystallography^{1-3,7-11} verifies some theories (see ref. 12) and dashes others; most importantly it changes the road map for future experiments. The new papers¹⁻³ are beautifully written and illustrated; those in the G-protein business should go directly to them to wallow happily in the details. Here I will highlight the main findings (about which both groups agree), and point out some differences.

The two groups used different approaches to alter G proteins slightly by mutating or removing lipid-modified residues in order to form crystals. The two approaches yielded the same general architecture and location of interfaces. The first surprise is that G_{β} is shaped like a 7-bladed propeller with a tapering, water-filled shaft-housing connecting the two faces (Fig. 2a). The G_{γ} subunit is draped like a thread along the side and over the

face of the G_{β} subunit. Along the other side of the propeller, G_{α} stretches its amino-terminal, lipid-modified arm to a tether point close to G_{γ} (Fig. 2b). Another interpretation employs the same tether sites but rotates the heterotrimer by 90° (Fig. 2c). The two subunits lie side by side, like two ships docked at the same pier.

There are two major contact sites between G_{α} and G_{β} . The first is the tethered prow of G_{α} , the myristoylated or palmitoylated G_{α} amino terminus. The larger and more important site is the interface between the G_{α} switch regions and one of G_{β} 's electronegative faces. The apposition of the two subunits in this region shields this vital domain, protecting the G_{α} face until the cationic lever of the receptor 'flips' the switch domain and triggers the G_{α} conformational change. After the receptor catalyses the Mg^{2+} -dependent replacement of GDP by GTP in the G_{α} helical/ G_{α} GTPase domain cleft, the G_{α} switch region springs shut. The now active, compacted and presumably free G_{α} thus exposes itself and the freed, but unchanged, $G_{\beta\gamma}$ to interactions with effector molecules.

From previous data it seems that the carboxy terminus of G_{α} is the site of some G_{α} effector and receptor interactions. The sites of such interactions on $G_{\beta\gamma}$ are less well understood, but likely places to look will be in the coiled-coil G_{β} - G_{γ} regions and in the variable strands at the outermost edge of the propeller blades. Interestingly, whereas another β -propeller protein¹³, galactose oxidase, employs the propeller

shaft tunnel in its activity¹⁴, the $G_{\beta\gamma}$ shaft appears to be too small for entry of polypeptide chains. It is tempting to speculate that the G_{β} propeller makes an ideal scaffold for a variety of protein interactions, such as between GIRK1, CIR and $G_{\beta\gamma}$ ^{15,16}, and perhaps acts as a matchmaker between GRKs and their receptors⁶.

Once bound by active freed G_{α} , some effectors are known to speed up G_{α} 's rather anaemic catalytic rates by up to 100-fold. The fact that some effectors are acted on by both G_{α} and $G_{\beta\gamma}$ implies that another complex of G_{α} /effector/ $G_{\beta\gamma}$ may form, perhaps with $G_{\beta\gamma}$ again positioning and also activating or inhibiting the effector. Presumably effector binding increases hydrolysis of the GTP wedged in G_{α} 's cleft. Once GTP is hydrolysed to GDP, the switch region of G_{α} exposes G_{α} 's molecular grappling hooks to redock the $G_{\beta\gamma}$

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subunit until the cycle is repeated.

Differences between the two sets of studies lie in the crystallographic details. The higher certainty about atomic coordinates (lower *B*-factors), resolution between atoms (atomicity) and refinement of the Sigler–Hamm model allows them to be more specific about some detailed interactions (the G_{α} amino-terminal/ G_{β} interaction, the G_{γ} / G_{β} interaction, the glutamine-200 side chain). Most importantly, their additional crystallography on lone $G_{\beta\gamma}$ shows that $G_{\beta\gamma}$ does not change its conformation when freed of G_{α} . On the other hand, the Sprang–Gilman group employed an intact $G_{i\alpha}$ rather than a $G_{1\alpha}$ / $G_{i\alpha}$ chimera. Differences in interpretation of how the heterotrimer might sit in relation to the membrane are illustrated in Fig. 2b and c. The advantage of the model in Fig. 2c is that it places the only electroneutral G_{β} face (blades 1/7 region) against the membrane, preserves the

amino-terminal G_{α} and carboxy-terminal G_{γ} membrane tethers, and presumably places the G_{α} carboxyl receptor-interacting domain closer to the membrane.

What is left to do? The most important questions might be answered by making crystals of the extended complex, including receptor/ G_{α} (GDP)/ $G_{\beta\gamma}$, G_{α} (GTP)/effector or $G_{\beta\gamma}$ /effector. Of course the major stumbling blocks again lie in the lipidic interaction domains which are the least well resolved regions in the current studies. And it will be particularly difficult to create crystals of the transmembrane proteins, including all G-protein-linked receptors as well as many effectors. Meantime, the latest structural data will launch scores of mutagenic warriors to work out the functional details. □

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TAXONOMY

Where do rabbits and kin fit in?

Michael J. Novacek

ONE of the most stimulating developments in comparative biology is the convergence of interest in reconstructing phylogeny — the genealogical tree of life — from various perspectives. The players now include not only comparative anatomists and palaeontologists, but also those collecting data from genes and proteins.

This confluence of interests, however, does not always lead to compatibility. For example, the mammalian 'superorder' Glires, which comprises that most productively diverse of all mammal groups, the Rodentia (rats, mice, squirrels and innumerable others) and the Lagomorpha (rabbits and pikas) has been enthusiastically supported by morphologists dealing with diverse features of the skull, teeth, skeleton and fetal membranes¹. Molecular studies, however, fail to confirm this grouping, a discrepancy starkly emphasized in a study of protein sequence data described by Graur *et al.* (page 333 of this issue²).

The discrepancy over lagomorph and rodent affinities is a facet of an intriguing evolutionary problem. One of the notable events in vertebrate history is the starburst of lineages that produced a great radiation of more than 20 living and extinct orders of placental mammals, the division that includes such diverse forms as whales and bats, and even our own primate relatives. This radiation seems to result from a phase of rapid diversification during the late Cretaceous and earliest Tertiary periods. Although it is logical that all these lineages did not undergo fission simultaneously, the time separating these splitting events was, geologically speaking,

relatively brief. Our added handicap of viewing events from the present, some 70 million years later, adds to the challenge of untangling the roots of the placental mammal tree. Development of a well-resolved phylogeny has been stubbornly elusive³.

But some sectors of the tree have instilled a sense of hope for resolution. Even early reviews emphasized the viability of lagomorph–rodent connection⁴. For all that there was some scepticism about this linkage⁵, more recent morphological work reversed the doubts. These orders have fossil records extending back some 60 million years to the Palaeocene, and the morphological clues found in both living and fossil rodents and lagomorphs point to affinities between them. Moreover, the fossil record increasingly suggests that both rodents and lagomorphs share a common area of origin and early evolution, namely Asia⁶.

In taking a fresh look at the question, Graur *et al.* analysed sequences from a variety of proteins and gauged the relative strengths of nodes linking lagomorphs with three other taxa. The first iteration involving Lagomorpha, Rodentia, Primates and an outgroup species is based on an impressive 88 protein sequences. The resultant four-pronged tree favours a closer grouping between primates and lagomorphs than between lagomorphs and rodents. Data were analysed under different methods of reconstruction and assessed for their reliability based on statistical resampling (bootstrapping).

The authors then attempted to find out

whether some other placental mammal order might be closer to lagomorphs than to primates. For this purpose, they conducted a series of four-taxon analyses in which a 'test' mammal is thrown into the batch. The prospective competitors include carnivores, hyraxes, insectivores, bats and a variety of other forms. Unfortunately, evidence in many of these successive analyses is not so comprehensive — fewer than 10 protein sequences are available in 11 of the 16 tests conducted. All of the tests except those incorporating flying lemurs (Dermoptera) and tree shrews (Scandentia) favour, with various levels of confidence, an affinity between primates and lagomorphs.

But do these analyses spell the death knell for the Glires supergroup uniting Lagomorpha and Rodentia? There are reasons not to rush to such a conclusion. The data represent only one of several lines of evidence, both morphological and molecular. True, many gene-sequence studies do not closely cluster rodents and lagomorphs³, but they are largely ambiguous and inconsistent in their support for groupings above the level of order⁷. Moreover, analysis⁸ of the nuclear gene for interphotoreceptor retinoid binding protein supports a lagomorph–rodent grouping. What is needed is a comprehensive summary of the sequence information derived from a suite of genes that would complement the protein study offered by Graur *et al.*

That requirement also bears on another dimension of sampling, one that raises questions concerning the four-taxon analysis employed in this and many other molecular studies. Although this small taxonomic coverage makes analysis more straightforward and enriches the sample of character information, it suffers from failure simultaneously to analyse the possible branching arrangement in phylogenies of all the relevant groups (in this case orders of mammals).

The problem is particularly serious in the case of placental mammal phylogeny because the overall phylogenetic tree is so poorly resolved. Simply put, the quartet analysis does not eliminate the possibility that data supporting a particular node (say primates plus lagomorphs) are indi-

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cations of false common ancestry. One of these groups could, after all, be more closely allied with some other taxon (for example, flying lemurs), but that cannot be ascertained unless all the relevant groups are analysed simultaneously. In this regard, it is noteworthy that morphological studies overwhelmingly deal with a large suite of taxa (certainly more than four) analysed with the maximum parsimony approach. It seems that the most insightful comparisons between molecular and morphological data will involve comparable methods and numbers of taxa⁹.

Finally, what of the morphological basis for the superorder Glires? Graur *et al.* dismiss the character evidence from this domain, suggesting that the false indicators are the result of misjudged polarities and parallelism. They claim that such morphological constraints are the direct result of the reliance on ancestral morphotype. However, the morphological characters supporting Glires could emerge from simple optimization procedures based on character distributions in outgroups and ingroups in ways analogous to molecular approaches. In reality, the com-

parison between these molecular results and morphological evidence have more to do with the unexplainable inconsistencies in phylogenetic data than the view that the morphological signal is simply wrong. Be that as it may, Graur and colleagues' results are provocative and will doubtless fuel the multidisciplinary passion for higher mammalian phylogeny. □

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GEOLOGY

The 5-billion-dollar bumps

Marcia McNutt

THE scientific community has benefited from a number of investments by the US Defense Department in new technologies, most notably the Internet, through which nearly all long-range scientific communication is accomplished, and the global positioning system, used for such varied purposes as measuring the drift of continental plates, the water content of the troposphere, and the acceleration of aircraft. We can now add to the list one more peace dividend: the release of the previously classified Geosat altimetry data. These data provide an unprecedented view of undulations in sea height by measuring the round-trip travel time of radar pulses between the satellite and the reflecting ocean surface. Because the sea surface conforms approximately to an equipotential, this information can be used to recover gravity anomalies over the world's oceans, caused by the fault scars recording the past and present motions of the plates, by undersea volcanoes and by other submerged or buried geological structures. By a conservative calculation, the data obtained from the \$80 million Geosat satellite over 18 months would have cost \$5 billion to collect using a conventional surface ship operating continuously for a century.

The release of this data set goes a long way towards rectifying one of the great embarrassments to Earth scientists, namely that we know the shape of the solid surfaces of the Moon, Venus and Mars better than we know that of our own planet. This situation persisted because the oceans are virtually impenetrable by all the frequencies of electromagnetic radiation used by spacecraft to image planetary surfaces. Measuring the gravity field with Geosat is not equivalent to measuring the depth of the oceans, but because gravity anomalies are caused by lateral changes in density, and the position of the rock/water interface at the sea bottom makes the largest contribution to lateral density variations,

the gravity field mimics variations in ocean depth.

The value of these data is enhanced by the fact that researchers from the Scripps Institution of Oceanography and the National Ocean and Atmospheric Administration have gone through the complex and laborious process of converting the raw round-trip travel times of the Geosat radar pulses into a gravitational field, and placed the results in the public domain, accessible over the Internet via anonymous FTP¹. The data are easily manipulated and imaged using the Generic Mapping Tools, a software package similarly available².

Figure 1 compares the best global bathymetric grid (a) with the Geosat gravity grid (b) for a portion of the South Pacific which includes the Cook–Austral islands. Both data sets are plotted in a transverse Mercator projection. The Geosat map reveals a large number of uncharted submarine volcanoes associated with the island chain. Furthermore, volcanism at the southeastern end of the chain occurs not as individual conical edifices, but rather along a sinuous, asymmetric ridge flanked by a trough to the south. This signature is typical of environments where plates are being pushed together or pulled apart. The southern Austral islands, therefore, might be aligned with cracks caused by intra-plate stress, rather than by a plume of melt ascending from the deep mantle (the prevailing view). Here and elsewhere, the

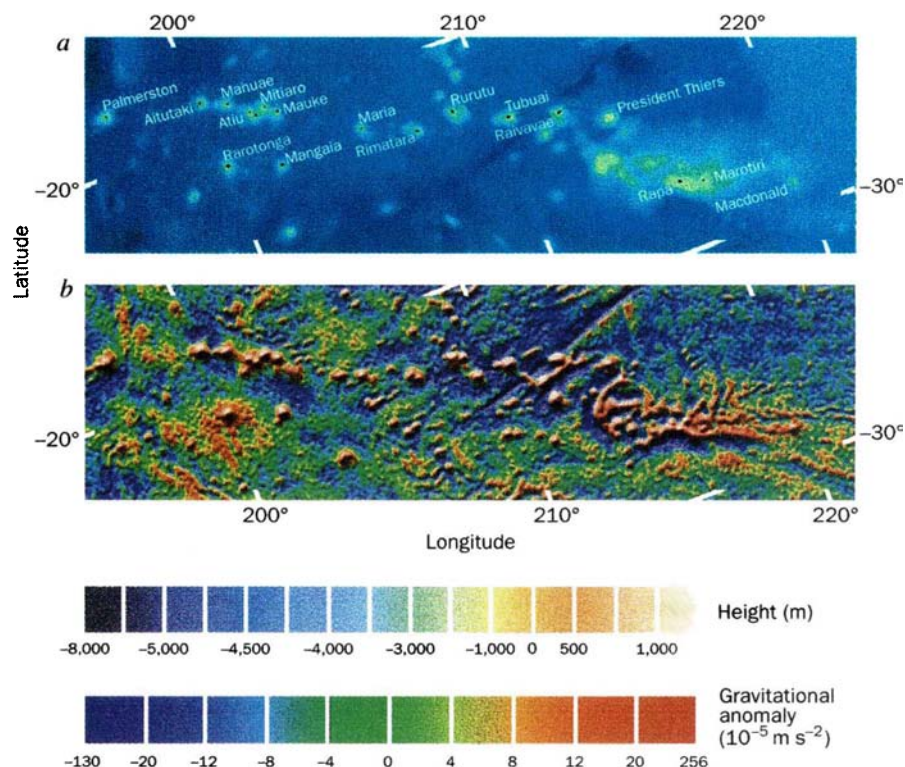


FIG. 1a, Conventional depth map of an area around the Cook–Austral islands region, in the Pacific. b, Geosat gravity data, showing their much higher resolution.

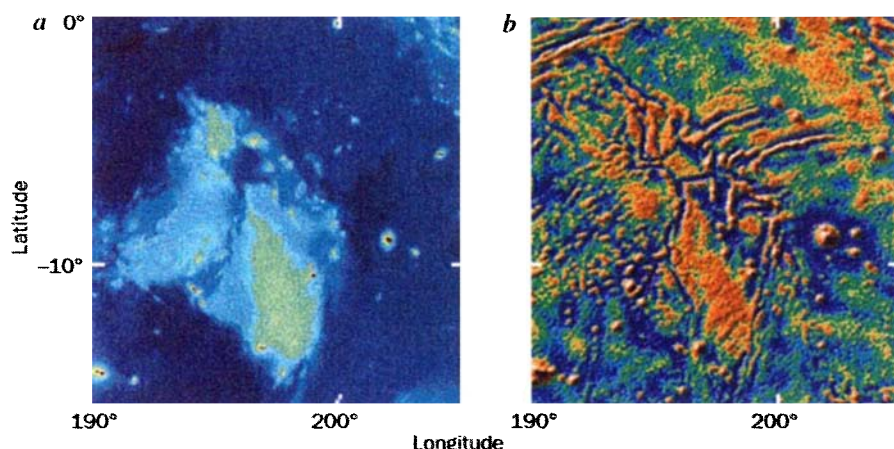


FIG. 2a, Bathymetric map of the Manihiki plateau. b, Geosat gravity reveals ancient plate boundaries around the plateau, which may have been formed by its eruption.

Geosat data reveal features of mid-plate volcanic activity not predicted by current plate tectonic theories.

In regions where ocean depths are relatively well known, the new Geosat data provide complementary information on buried geological structures. For example, the shape of the Manihiki plateau (Fig. 2a) follows the traces of ancient plate boundaries hidden beneath volcanic flows and thick sediment piles, but now apparent as sharp lineations in the Geosat gravity field (Fig. 2b). Preserved in this pattern is the history of plate reorganization that more than 100 million years ago led to the establishment of the system of mid-ocean ridges responsible for the creation of most of the present-day Pacific sea floor. The fact that Manihiki is superimposed on this reorganizing plate boundary may be significant. One possibility is that the huge amount of heat involved in the eruption of the plateau weakened the plate so much that new sites of seafloor spreading formed on the plateau margins, as old ones were abandoned.

Geosat is just one in a series of radar altimeter missions launched by civilian and defence agencies. In the 1970s, NASA's Geos-3 and Seasat satellites provided the first global view of the marine gravity field³, and demonstrated to the marine science community the value of this information for discovering uncharted features, tracing tectonic lineations and measuring the thermal and mechanical properties of the plates. However, the resolution of the first gravity maps was only 100–200 km because of the short lifetime of these missions. Seasat returned truly spectacular data, but failed only 3 months after launch, prompting rumours that the satellite was intentionally sabotaged because it 'knew too much'. However, a civilian inquiry panel determined that the cause of failure was a short circuit in a component that a contractor had not properly tested for space flight. Nevertheless, it was obvious to the military establishment that the altimetric gravity data

would have strategic importance for the use of inertial guidance systems for precision navigation, and a security classification was imposed on all future US altimetry data, civilian or military, except along satellite tracks that duplicated the Seasat coverage.

The recent decision to release the Geosat data probably has less to do with the end of the Cold War than it does with the fact that the European ERS-1 satellite has returned publicly available altimetric data on the oceans, of comparable resolu-

tion and accuracy to that obtained by Geosat nearly 10 years ago. Although there may have been good reasons for keeping the Geosat data under wraps for so long, it is unfortunate that in the intervening decade untold millions of civilian dollars have been spent on efforts to obtain information already in hand but locked away. In 1991, I spent half a million dollars in ship time mapping a tectonic lineation in the South Pacific that is strikingly evident on the new Geosat map. The military still has a security classification on a number of other data sets of questionable strategic value, such as marine magnetic data, depth soundings and sediment thickness measurements. It is to be hoped that these enormously valuable data sets will be declassified soon, so as to maximize the return on our investment in marine research. □

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PHOTOBIOLOGY

Wrinkles waiting for G₀T

Douglas E. Brash and Barbara A. Gilchrest

BABY bottoms are smooth. So, in fact, are octogenarian bottoms — the wrinkles, 'pebbles' and much of the lost elasticity we associate with ageing are limited to skin exposed to sunlight. These changes seem to result from tangles of disrupted collagen and abnormal elastin in the dermis, together with loss of normal collagen and elastin and the fibroblasts that make them. In sun-shielded skin, the losses are much slower and the abnormal collagen and elastin are absent¹.

This overlap of findings has caused sun-induced skin damage to be considered a partial ageing syndrome, and so skin provides a rare opportunity to separate ageing's environmental and internal biological components. On page 335 of this issue, Fisher *et al.*² report what may be a molecular mechanism for some of the degenerative changes of photoaged skin — the breakdown, by enzymes induced by ultraviolet radiation, of collagen and elastin. They also present possible treatments.

It has never been clear how many of the body's senescence pathways result from cell loss and how many from cell malfunction. Work with tissue cultures has, however, provided considerable

understanding of loss of proliferating cells. After growing for about 50 population doublings, cells arrest in the G₀ stage of the cell cycle; they can then survive for years without dividing. Cells from older patients or those with premature ageing syndromes undergo fewer doublings.

The arrest at G₀ turned out to be associated with increased expression of genes such as *SDI-1* (ref. 3), an inhibitor of cell-cycle-coupled protein kinases also cloned as the growth-suppression gene known as *WAF-1*, *CIP-1* or *p21*, that can be induced by the tumour suppressor p53. It also emerged that telomeres, the 'caps' at the ends of chromosomes, shorten as cells are grown in culture or as patients age, with the residual length apparently determining the number of population doublings remaining⁴. Telomerase, an enzyme that allows telomeres to be re-extended, is active in germ cells and most tumours. These findings have suggested that senescent G₀ arrest is triggered by a genetic clock.

Fisher *et al.* have taken a different tack, focusing on cell malfunction. Earlier experiments on the effect of ultraviolet radiation on cells, far above the lethal dose (D₀), and using wavelengths not

found in sunshine, showed that cells in culture activate signalling mechanisms termed the 'UV response' which involve pathways running from a receptor at the cell surface, through the Ras and Raf proteins, to the gene-transcription factors AP-1 and NF- κ B (ref. 5). Below the lethal dose, fibroblasts, keratinocytes and melanocytes irradiated with ultraviolet-B (UVB) increase expression of p53 and p21; cellular consequences are cell-cycle arrest and programmed cell death^{6,7}.

Stunningly, the new results² — among the first such data from intact human skin — reveal that several protein-degrading enzymes (proteases) are induced by UVB exposures far below the lethal dose, even at a tenth to a hundredth of that required to give a minimally detectable sunburn. These exposures correspond to 2–3 minutes of summer sunshine.

The authors first found 6–60-fold induction of the messenger RNAs for interstitial collagenase, stromelysin and 92K gelatinase, enzymes that degrade collagen and elastin during wound repair. The corresponding protease activities were also increased. A 72K gelatinase was not induced. Because the three induced genes had a recognition site for the AP-1 transcription factor, which can regulate them *in vitro*, the authors then used gel-shift assays to show that low UVB doses induce AP-1 in skin. UVB also induced NF- κ B, an *in vitro* regulator of the 92K gelatinase. Inhibitors of AP-1 induction largely blocked induction of these matrix-degrading enzymes.

Because reduced dermal collagen and abnormal elastin are prominent features of sun-damaged skin, the authors suggest that chronic exposure to sunlight slowly degrades the matrix proteins of the dermis, leading to wrinkles and elastosis. If so, prevention of photoageing seems a reachable goal. The inhibitors of AP-1 induction used by Fisher *et al.*, retinoic acid and a glucocorticoid, are in fact already used as drugs. Retinoic acid, administered under medical supervision, has been shown to reverse unwanted changes in photoaged and normally aged skin^{1,8}. The degree to which protease induction is inhibited may predict an individual patient's response to long-term retinoid treatment. Induction itself may predict individual vulnerability to sun-damage. Retinoic acid also ameliorates skin cancers and precancers⁹, so low doses of UV may have similar effects on genes regulated by AP-1 and NF- κ B relevant to epidermal cancer. These treatments will be selective for particular UV-induced signal transduction pathways, because Fisher *et al.* found that inhibiting AP-1 induction did not inhibit sunburn's skin reddening. Others have found that inhibitors of reddening do not inhibit programmed cell death or suppression of the immune system.

But we need to pause here. How, experimentally, can one confirm the tissue-level effect of a specific molecular

event that *in vivo* recurs over a timescale (T) of years? Degradation of collagen and elastin by proteases might well be a beneficial repair response, in which tissue remodelling removes damaged matrix proteins. The side-effects of retinoic acid, at least in current animal models, make it awkward to interpret long-term treatment of UV-irradiated skin, so it will be difficult to isolate causes.

Molecular biologists have had the luxury of almost black-and-white experiments, but data such as those of Fisher *et al.* now force us to confront long-term, low-dose effects. A similar situation occurs in the origin of skin precancers, which appear to exist in equilibrium between clonal expansion and regression of cells, with sunlight favouring expansion¹⁰. Whereas the initiated cell is created by a genetic change that is easy to measure, clinical manifestation as a precancer requires the chronic and repetitive forces to be played out.

The challenge will be to find tools for a more sophisticated era, just as geneticists have begun to tackle the subtlety of multi-genetic diseases by combining their DNA probes with mathematics originally developed for quantitative traits such as body height¹¹. The new weapons might include long-term studies on mice with specific gene knockouts; or analysing as a multi-genetic disease the population differences in patterns of tissue senescence. To short-circuit the long time spans, comparative evolutionary models have been used to identify correlates of lifespan in a series of mammalian species. Constructing transgenic mice in both long- and short-lived species may be the ultimate approach.

Whatever the tools, they are likely to be of general importance — it seems inescapable that ageing, like precancerous conditions and perhaps atherosclerosis and diabetes¹², reflects the summation of small but recurrent assaults on the cells of the body. Unlike Godot, G₀D₀T eventually arrives. □

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Tuned heat

ALMOST every engineering component is tense with locked-in stresses. Casting, stamping, moulding, machining, all leave the product rather like a wound-up spring. As a result, everything slowly creeps, even cast iron. The main casting of a lathe must be left for at least a year before final machining; otherwise it will creep in service. Big lasers are often mounted on slabs of natural granite, which has had millions of years to relax its casting stresses.

Another way to relax a material is annealing: heating it up and letting it cool slowly. It shakes down the atomic lattice very inefficiently, and can also introduce contractile stresses when it is withdrawn. Heat, of course, is the random battering of a solid lattice by thermal phonons of all energies. What is needed, says Daedalus, is a sort of 'tuned heat': a blizzard of monochromatic phonons all of the same energy. They would be tuned to just that energy needed to mobilize the lattice defects which lock in the stress. Bathed in the warm glow of a correctly tuned heat, any component would relax completely and anneal perfectly.

So DREADCO physicists are exploiting modern cathode-ray tube and mass spectrometer technology to build a novel electron gun. It will fire electrons with a precisely defined energy, or set of energies. When they hit the surface of a solid, their identical impacts will launch identical, monochromatic phonons into it. These will skitter around the lattice, relaxing the dislocations they are tuned to. With luck, each dislocation as it relaxes will release its surplus energy as another phonon of that energy. A sort of phonon avalanche will spread through the lattice, relaxing it swiftly and completely. To test the process, the team will dissolve its products in nitric acid, and measure their heat of solution. Fully relaxed components, completely free of internal energy, will give out far less heat than the tense, stressed products of traditional metal-bashing.

Tuned heat will usher in a new era of engineering. Nuts, bolts, girders, panels, all will be freed from their hidden tensions. Relaxed and confident, they will at last accept loads up to their full strength. Brittle failure, fatigue and even rusting, all of which usually start at some highly stressed site, will vanish too. Lighter, stronger, more reliable designs will be possible. A portable tuned heater could even relax existing stress-burdened structures *in situ*. Old cars and bridges, and aircraft reaching the end of their fatigue lifetimes, will be swiftly rejuvenated. Released from their besetting worry, engineers will relax too.

David Jones

Kuwait oil lakes as insect traps

SIR — During the Gulf War in early 1991, Iraqi occupation forces blasted oil wells and pipelines in the desert of Kuwait, forming hundreds of oil ponds. These still exist¹, and continue to trap a variety of different animals². From October 1994 to May 1995, one of us (J.Z.) regularly visited two ponds north of Kuwait City, at Al-Muddayarah, along the road to As-Subiyah (47° 55' E, 29° 40' N)². Reductions in the oil level due to evaporation and percolation into the ground created distinct bands of insect carcasses at their edges. Bands of dead dragonflies, damselflies and ground beetles may reflect arrivals of migrating insects in autumn and spring. We witnessed such arrivals of aeshnid dragonflies in October 1994 and February 1995, many females being trapped while attempting to lay eggs in the oil. We suggest that these insects are attracted by the strong polarization of light reflected from these pools.

Oil lakes trap different animals in different ways. Terrestrial animals become entrapped during foraging or migration, or they may be attracted to the water that overlies the tar during winter and spring. Water-seeking birds and many flying insects land directly on the surface or at the edge of the oil lakes. Water insects can detect water by means of the horizontal polarization of light reflected from the water surface^{3,4}, and while some insects may crash into the oil, others are clearly attracted to the lakes.

We have compared the polarization characteristics of crude oil and transparent water surfaces by video-polarimetry (*a* in the figure). Oil shows a higher contrast in intensity than water between shaded and diffusely illuminated regions. Light emanating from water is vertically polarized when refracted light dominates⁵ (*a* in the figure, top half of dish) and horizontally polarized for surface-reflected light^{6,7} (bottom half of dish), but light is not diffracted from oil, penetrating light being absorbed by the dark pigments. Light from an oil surface is therefore always more or less horizontally polarized. For insects that see in the ultraviolet, oil surfaces closely mimic the polarization and reflectivity

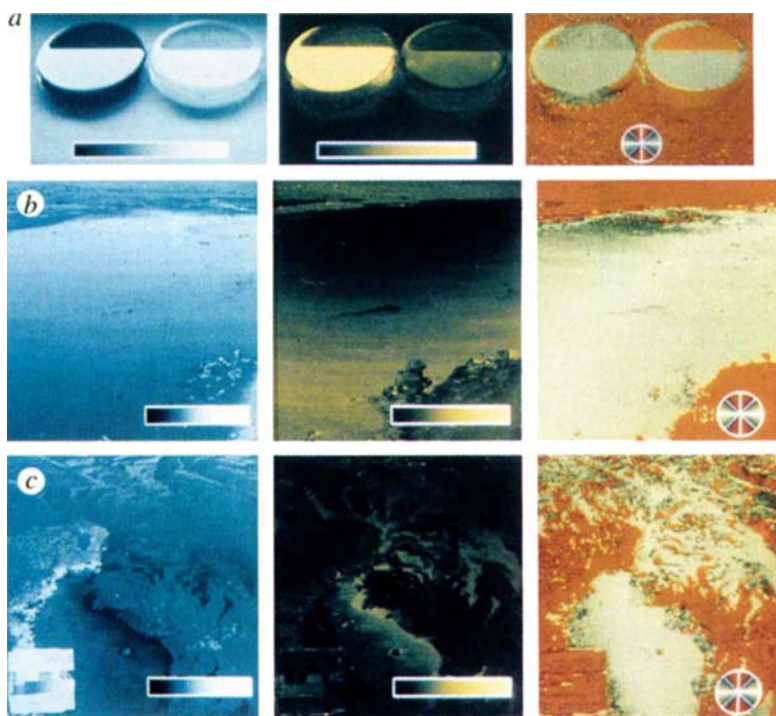
characteristics of water, differences in transparency being restricted to the visible spectrum. Even for animals sensitive to polarized light at visible wavelengths, oil lakes could appear as exaggerated water surfaces (supernormal stimulus).

The surfaces of real oil lakes, in both summer and winter (when rainwater and oil form complex surface features), are highly polarized, whereas the degree of polarization of sand-covered tar is very low (*b, c* in the figure). The direction of polarization is horizontal, while areas with rough surfaces reflect vertically polarized light. The high degree of polarization where oil and water meet (*c* in the figure, centre) again demonstrates the effect of transparency: the sandy bottom lies in the shadow of a floating oil slab and horizontally polarized reflection thus dominates. The degree of polarization is reduced in bright areas of water puddles (*c* in the fig-

ure, lower left), because the polarization of surface-reflected light is degraded by the refracted, vertically polarized light returning from the sandy bottom.

The Kuwait oil lakes can be considered as an upscaled version of Schwind's experiments^{3,4}. As disastrous as they are, they offer unique research opportunities. They are massive animal traps that could be used to monitor the distribution, seasonal occurrence and movements of desert animals. Because they also mimic water surfaces, they attract water insects and birds on migration that are otherwise difficult to record, and could help to monitor changing migration patterns caused by the draining of marshlands in southern Iraq.

Like the contemporary oil lakes in Kuwait, the palaeontologically important Rancho La Brea tar pits at Hancock Park in Los Angeles⁸, and tar pools at Starunia in western Ukraine^{9,10}, trapped a variety of water insects and birds during the Pleistocene epoch. Most animals found at Rancho La Brea and Starunia are thought



a, The polarization characteristics of crude oil (left dish in each picture) and water (right) measured with a video-polarimeter. The top half of the dishes was in the shadow, the bottom half was illuminated by unpolarized diffuse light from an overcast sky. Insets show the grey-level and colour codes for intensity, degree of polarization and **E**-vector alignment. Left, brightness distribution. Brightness, *I*, varies from black (*I* = 0) to white (*I* = 255). Centre, degree of polarization, *d*. Black, *d* = 0%; bright yellow, *d* = 100%. Right, direction of polarization. Red symbolizes vertical, green, horizontal **E**-vectors. Viewing direction was 55° to the vertical. *b*, Reflection-polarization pattern of oil lake on 9 May 1995 looking north and at 75° relative to the vertical. The surface consisted of smooth, clean, homogeneous, low-viscosity oil. *c*, Oil lake on 15 January 1995 looking east and at 70° relative to the vertical. The surface is not smooth. Rainwater has accumulated during the winter months; slabs of oil swim on the surface of the water and sand has settled on some of them. The scenes were filmed with a video camera through a linear polarizing filter which was turned in 15° steps during recording. The films were subsequently digitized frame-by-frame and the modulation of the intensity was determined from a series of video pictures as a function of the alignment of the polarizer.

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to have stumbled accidentally across these tar pools, but we suggest that some animals were deceived by and attracted to the pits by the strong reflection-polarization of the oil surface mimicking a body of water.

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Hidden quasars reddened by dust?

SIR — Webster *et al.*¹ argue for a large population of dust-reddened quasars, on the basis of the optical-infrared colour diversity in their new sample of radio-loud quasars with flat radio spectra. If their sample is representative of the quasar population, then their results imply that optical surveys miss about 80% of quasars, and that these missing quasars could account for the observed X-ray background. We argue that there is a simple way of avoiding these radical conclusions. Their results are influenced by an additional red, optical synchrotron component peculiar to flat-spectrum radio-loud quasars, with its own resulting colour diversity caused by the range in relative contributions of the 'normal' quasar and synchrotron components.

Flat radio spectra in quasars are caused by enhancement of synchrotron emission from compact regions within a jet by relativistic beaming in the direction of its motion². Selecting by flat radio spectral index should therefore bias strongly in favour of quasars whose radio jets lie very close to the line of sight. In the context of Unified Schemes³ in which the jet emerges along the relatively unobscured poles of an anisotropic distribution of material, or in

which the jet itself clears material along its path, flat-spectrum quasars should be subject to very little intrinsic reddening.

It has been known for many years, however, that flat-spectrum quasars can have unusually red optical-infrared colours (for example, ref. 3). In several cases there is direct evidence from time variability that these colours are the result of a non-thermal source of red light which is superimposed on the normal blue quasar continuum (for example, ref. 4) and which is likely to be an extension of the synchrotron emission that dominates in the radio; many other studies also suggest the presence of a beamed optical-infrared component (for example, ref. 5). Indeed, many of the Webster *et al.* quasars are classified optically as blue stellar objects, despite their red optical-infrared colours⁶. This argues strongly against a reddening interpretation, but is consistent with a beamed, red synchrotron component.

Such a beamed component of optical emission may also explain the unusually large range of broad-line equivalent widths amongst flat-spectrum quasars (compare ref. 7 with Webster *et al.*'s Fig. 2; see also ref. 5). An observable anti-correlation of broad-line equivalent widths with *B-K* colour is not a firm prediction of the beaming model because the combined dispersion in the equivalent widths, the unbeamed continuum slope, and the slope of the (variable) beamed component may be very large. However, Jackson *et al.*⁸ have demonstrated an anti-correlation between the dominance of flat-spectrum radio

emission and the equivalent widths of the emission lines. This is strong evidence for an additional source of continuum emission in flat-spectrum quasars which, if red (as expected for synchrotron emission), provides a simple explanation for the colour diversity reported by Webster *et al.*

Some quasars may be reddened by the mechanism favoured by Webster *et al.* The well-known population of red quasars associated with steep-spectrum radio sources, in which the red beamed optical component is not expected to dominate, have broad lines sometimes only revealed by near-infrared spectroscopy⁹. Nevertheless, complete surveys at low radio frequencies¹⁰, which are insensitive to beamed objects, contain few red quasars, again suggesting that the incompleteness in optical quasar surveys is much less dramatic than concluded by Webster *et al.*

Perhaps a more pertinent question is whether narrow-line objects constitute a population of obscured quasars. Studies of steep-spectrum radio sources² are consistent with the hypothesis that narrow-line radiogalaxies are quasars whose nuclei are obscured by material with column densities orders of magnitude higher than those invoked by Webster *et al.*

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New photosynthesis or old?

SIR — Greenbaum *et al.*¹ have described the light-induced hydrogen and oxygen evolution in a mutant strain of the green alga *Chlamydomonas reinhardtii* lacking photosystem I (PSI). As the mutant cells consumed carbon dioxide in the light, Greenbaum *et al.* suggested that reduced NADPH (nicotinamide adenine dinucleotide phosphate, obligatory for carbon fixation and normally formed by PSI) was generated by the direct reduction of NADP⁺ by pheophytin, the low-potential electron acceptor of photosystem II (PSII)². The authors claimed "a new type of photosynthesis being performed by the PSII light reactions exclusively".

Transient photosynthesis has been reported from a *Chlamydomonas* mutant lacking PSI (for example, ref. 3), as has direct reduction of NADP⁺ by pheophytin in various PSII-enriched preparations (for example, refs 4, 5). The quantum yields were low in the latter case⁶. Apparently, the forward electron transfer from the reduced pheophytin to NADP⁺ was not competitive with its backreaction (occurring in a few nanoseconds⁷) with the oxidized primary electron donor of PSII. The

experiments with the PSI-lacking mutants, however, yielded a high rate of carbon dioxide consumption, comparable to that in wild-type cells. This high quantum yield may point to another mechanism of NADP⁺ reduction.

The photosynthetic reaction centre of purple photosynthetic bacteria is evolutionarily and functionally related to PSII⁸ (except for its inability to oxidize water). Bacteriopheophytin, with the same low redox potential as its counterpart in PSII⁹, does not directly reduce nicotinamide dinucleotides. Instead, they are formed by reversed electron flow through the NADH:ubiquinone oxidoreductase complex. The driving force of the reversal consists of the scalar reducing potential of the ubiquinone/ubiquinol redox pair plus the electrochemical potential difference of the proton across the photosynthetic membrane¹⁰. *Chlamydomonas reinhardtii* incorporates an active NAD(P)H:plastoquinone oxidoreductase in its thylakoid membrane^{11,12}. Thus, we propose a more traditional interpretation of the data by Greenbaum *et al.*, namely, that electrons provided by PSII first reduce the plasto-

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quinone pool and only then does the protonmotive force drive them into NADP⁺. Both conflicting hypotheses can be discriminated by applying uncouplers and plastoquinone antagonists. Until the necessary tests have been performed, the question of an efficient, physiologically relevant, direct reduction of NADP⁺ by PSII, and hence the challenge to the general validity of the Z-scheme of Hill and Bendall¹³, remains unsettled.

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GREENBAUM ET AL. REPLY — In our Letter we reported a new phenomenon, CO₂ fixation and hydrogen and oxygen evolution, at wild-type rates, in a mutant alga that lacked the photosystem I reaction centre. We did not, however, attempt to elucidate the mechanism or the pathway of this discovery, or claim evidence for “the direct reduction of NADP⁺ by pheophytin...”

Mulikidjanian and Junge speculate that the mechanism of ‘PSII photosynthesis’ is reversed electron flow through the NAD(P)H:plastoquinone (PQ) oxidoreductase in the thylakoid membrane, as previously advanced by Peltier and Thibault¹⁴ to explain electron transport in isotopic oxygen exchange experiments in F18, another PSI-deficient mutant of *Chlamydomonas reinhardtii*. We considered this mechanism, but we are convinced that this explanation is not correct. Antimycin A is known to inhibit chlororespiration by blocking electron transport between NAD(P)H and the PQ pool which is mediated by the thylakoid membrane-bound NAD(P)H-PQ oxidoreductase^{15,16}. Our recent experiments (J.W.L. and E.G., manuscript in preparation) indicate that antimycin A has no effect on PSII photosynthesis.

In addition, we have found that 5 µM FCCP (carbonyl cyanide trifluoromethoxy-

phenyl hydrazone) completely inhibits CO₂ photoassimilation but increases the sustained simultaneous photoevolution of molecular hydrogen and oxygen. FCCP is a protonophore that dissipates proton gradients across the photosynthetic membrane and thus inhibits synthesis of ATP, which is essential for CO₂ assimilation by the Calvin cycle but not essential for hydrogen production by the ferredoxin/hydrogenase pathway. It is known that the reverse-operating NAD(P)H:PQ oxidoreductase is driven by a proton gradient across the thylakoid membrane. If such a reverse-operated mechanism were responsible for electron transfer from PQH₂ to ferredoxin, FCCP would inhibit not only CO₂ assimilation but also H₂ photoevolution, because both are dependent on electron transfer from PQH₂ to ferredoxin. FCCP's effect cannot be explained by the reverse-operating NAD(P)H:PQ oxidoreductase mechanism.

Moreover, we have demonstrated that PSI-deficient green algae can grow photoautotrophically using CO₂ as the sole source of carbon, light as the sole source of energy, and water as the sole source of electrons under both aerobic and anaerobic conditions in a minimal medium (water plus mineral elements but without organic nutrients; J.W.L., C.V.T., L.J.M., T.G.O. and E.G., manuscript submitted). Photoautotrophic growth and the quantum requirement of photosynthesis in PSI-deficient mutants (E.G., J.W.L. and C.V.T., manuscript in preparation) preclude the reverse-operating NAD(P)H:PQ oxidoreductase from being responsible for PSII photosynthesis.

Mulikidjanian and Junge state that their proposed mechanism uses the scalar reducing potential of the ubiquinone/ubiquinol redox pair plus the electrochemical proton potential difference to drive the reduction of NADP⁺. Even if the operation of that mechanism can generate NADPH, it will leave little or no proton-gradient energy for synthesis of ATP, which is required for CO₂ fixation by the Calvin cycle as well as for cell growth. This mechanism cannot, therefore, explain our newly discovered PSII photosynthesis and its support of photoautotrophic growth. We believe that the mechanism of PSII photosynthesis involves electron flow from PSII to ferredoxin/NADP⁺ reduction through the plastoquinone pool and cytochrome *b/f* complex (J.W.L. and E.G., manuscript in preparation).

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Lateral proton diffusion

Sir — The lateral diffusion of protons along membranes could provide a direct link between sources and sinks involved in chemiosmotic coupling¹. Recently, a long-distance migration of protons along membranes has been observed in purple membranes and reconstituted bacteriorhodopsin²⁻⁵. This was suggested to be due either to protonation/deprotonation reactions of amino groups or polar headgroups of lipids, or to a movement along interfacial water molecules²⁻⁵. Scherrer has suggested that evidence for a lateral movement of protons along a surface could be obtained by modulation of the chemical character of the lipid headgroups⁵. The dwell time of protons depends on the lipid headgroups⁶, and so this is expected to control any lateral proton movement. Studies on dissociation rate constants concluded that surface-to-bulk proton transfer was not retarded and so there was no lateral proton movement^{7,8}.

The experiments proposed by Scherrer have already been performed in lipid monolayers, by comparing long-range movements of protons from a source to detectors either at the membrane level or in the bulk medium (as reviewed in ref. 9). Lateral migration of protons is observed with many phospholipids as long as the molecular assembly is in the fluid state, and is therefore not controlled by the chemical character of the polar heads. Rather, the migration of protons along membranes may be supported by a hydrogen-bond network involving polar headgroups and interfacial water molecules. A ‘hop and turn’ mechanism would be involved and controlled by the correlation time of the lipid headgroups, as experimentally observed, and not their chemical character. As soon as the continuity of the network is broken (for physical or chemical reasons), the lateral migration of protons is prevented.

Migration was critically controlled by the composition of lipid/detergent mixed

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films¹⁰ which alters the conducting network (as shown with CTAB in phosphatidylethanolamine), in agreement with the results on reconstituted bacteriorhodopsin²⁻⁴.

It is clear that a thermodynamic barrier prevents free proton movement from the surface to the bulk, as proposed 10 years ago¹¹ and confirmed by the studies on purple membranes²⁻⁴.

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SCHERRER REPLIES—Teissie refers to earlier work on a phospholipid monolayer system. The interpretation and the model proposed for diffusion of protons along the membrane surface are basically in agreement with our data from the bacteriorhodopsin-lipid-detergent system³ and data from the purple membrane system^{2,4}.

The experiment I proposed⁵ to obtain direct experimental evidence for lateral movement of protons requires a proton source on the membrane surface, proton detectors in well-defined positions on the

membrane surface (at the proton source and a defined distance away), and a proton indicator in the aqueous bulk medium. In the experiment referred to by Teissie the proton source was in the bulk medium and the proton indicator in the monolayer, and so that system does not meet these requirements.

In addition, in our studies on bacteriorhodopsin-lipid-detergent micelles³, we showed direct experimental evidence for the effect of the pK_a of lipid headgroups on the lateral transfer of protons, and for the delayed proton transfer from surface to bulk. The conclusion drawn from previous studies on the dissociation rate constants of the lipid headgroups against a retarded proton transfer from membrane surface to bulk^{6,7} does indeed not agree with our experimental data. However, this conclusion was based on data obtained in a model system that again did not allow the defined placement of the proton source and proton indicators.

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Chaos in the classroom

SIR—It is said that many universities in the UK struggle to fill undergraduate science places, asking lower entry grades than other subjects. *The Independent* newspaper recently suggested that sciences are "considered intellectually difficult and not always well taught in schools"¹. Could school science be failing to fire students' enthusiasm? I recently completed my secondary school science education in an excellent tertiary college,

but found parts unimaginative and too focused on factual recall. Nuffield A-level physics, however, offered real opportunities in course work to escape the well-worn and mundane if one wished. Here I present my work, based on the dripping tap as a model chaotic system, to illustrate possibilities inherent in simple experimental investigations for inspiring students to study science.

A system of three plastic header tanks,

Scientific Correspondence

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rubber tubing (donated by The Leyland Rubber Company) and a small pond pump provided a constant but variable pressure, isolated from vibration. Water dripped through a capillary tube, and drops were detected using a light-dependent diode (LDD), allowing measurement of the time gaps between each successive drop. Observation of the relationships between these time gaps revealed a number of potentially chaotic behaviours with different head heights (see figure). As would be expected, with increasing water pressure the average time between drips decreases, but not in a linear manner.

Statistical analysis was simplified because the system is discrete. Tests of randomness² showed that all times related to previous times. Shaw defined "information stored in a system" as the difference in its expected randomness with and without knowledge of its past³. In tests for this, Markov chain χ^2 analysis of the 5.8-cm data proved very significant for first-order relationships (strong evidence for long/short dipolarity, a relatively long gap being followed by a relatively short gap) and quite significant for second-order relationships (gaps affect the next but one gap). Serial autocorrelation² of the data turned up many other strange relationships; for example, in the 4.8-cm data, gaps appeared unrelated to the immediately preceding gap but were related to all previous gaps; in the 5.8-cm data, an autoregressive process⁴ showed that there was a large jump in stored information with the tenth preceding gap.

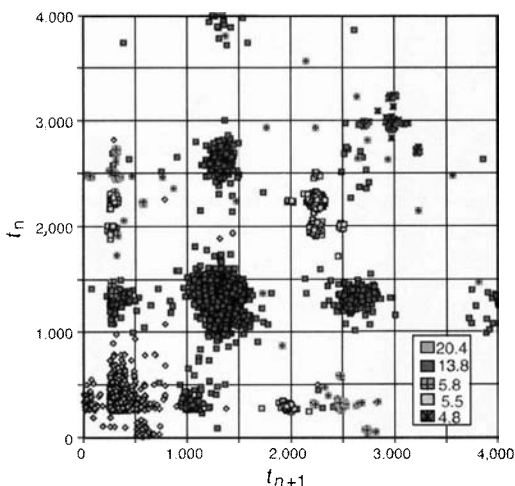
Far more data were produced than could be analysed in the three weeks allowed for the project. But the innovation involved in solving the problems of experimental design and data analysis (thereby acquiring skills from computer programming and electronics to surface tension and plumbing) was similar to 'real' research. Experimental investigations are the bedrock of all scientific discovery; surely there must be many students, like myself, in all branches of science, whose imaginations would be fired by encouraging them to do less 'well-worn' and more innovative work?

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1. *The Independent* 2 Nov., Sect. 2, p.13 (1994).
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Return maps (plots of n th against $(n-1)$ th time between drips) of five different types of behaviour observed at different head heights. More than 12,000 data points were recorded, many superimposed, so that the 'noise' is less than appears at first glance. The 4.8-cm data had a single period with subgroups, the 5.5-cm data show short and long time periods, while the 5.8-cm data are dipolar (that is, for example, short, long, short). The 13.8-cm data have four time periods (a further bifurcation doubling) and had the greatest number of definite time periods found, whereas the 20.4-cm data have progressed to a more independently random system (with the square left-hand corner to the main group showing the definite end of phase space) which has only two obvious time periods.



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SEMI-HELMETED hornbill from *A Monograph of the Bucerotidae, or Family of the Hornbills* by Daniel Giraud Elliot (1882). Now published by Fundacef, DM450 (see page 307).

found living birds a congenial subject", but that it is unlikely that as a consequence of writing the play he would "eat birds with any less gusto than before". We should not anachronistically idealize or sentimentalize his attitude to them, or overlook the element of fantasy. (Similarly, other plays

by him which envisage women as ending Greece's civil war by a male-subduing sex-strike or as themselves seizing political power are sublimely comic unrealities, not a precocious comprehension of the then non-existent concept of women's rights.) But ethology has shown how the very process of hunting (or sacrificing) animals can create a sympathy towards them on man's part, and the increasing popularity of this drama today is surely very largely connected with its theme of man's relationship to the animal world. *Birds* is Aristophanes' longest surviving play, and this new edition is the longest commentary on any of his plays, not least because of the ornithological identifications it requires. Possibly the best compliment I can pay it is to say that I felt only one word was superfluous, the disyllable in the statement on p.14 that "*Birds* is perhaps Aristophanes' masterpiece". □

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How many people can we support?

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The Stork and the Plow. By Paul R. Ehrlich, Anne H. Ehrlich and Gretchen C. Daily. Grosset/Putnam: 1995. Pp. 364. \$30.

Who Will Feed China? By Lester R. Brown. Norton/Earthscan: 1995. Pp. 163. \$19.95 (hbk); £9.95, \$8.95 (pbk).

SOLUTIONS to the problems of the scarcity of resources facing human populations have been characterized by Joel E. Cohen as belonging to one of three classes: bigger pie, fewer forks or better manners. Paul and Anne Ehrlich have generally been associated with the 'fewer forks' school — but *The Stork and the Plow*, written with Gretchen Daily, suggests that they are moving towards 'better manners' remedies: as much weight is given to fairer world trade, balancing the interests of rural food producers and urban consumers, and sexual equality as to discussion of the slowing of population growth and reduction of resource consumption. Morally I find myself in broad agreement with these priorities, but I am left worrying about the implied assertion that such solutions are based on scientific rationale rather than ethical choice.

The authors' main argument is that, left unchecked, population growth and growth of consumption will endanger the life-support systems of our biophysical environ-

ment, but that control of these two threats can be established and sustained only in a globally fair and just society. The well-known negative correlation between female education and fertility level is repeatedly used to illustrate how equity works to the advantage of ecological balance, and there is a well-reasoned account of how poverty forces unsustainable use of natural resources — hungry peasants eating their seed corn is the classic example. These themes are familiar to social scientists working in this field, but their restatement by prominent natural scientists indicates that the intellectual gulf between the views on these problems of economists and ecologists is gradually being bridged.

The book is aimed at nonspecialists, and the authors tackle a broad range of factors with a bearing on growth in animal and human populations — such as migration, nuptiality, homosexuality and infanticide — but with little attempt to quantify their effects. Occasionally, quantitative estimates based on rather naive extrapolations are presented uncritically (for example, the global contribution of induced abortion to maternal mortality). The denunciation of the attitude of the Roman Catholic hierarchy to the regulation of fertility is quite entertaining, and there are some revealing insights into double dealings between the Reagan administration and the Vatican, and between the agrochemical industry

and present members of the US Congress. There is a careful and balanced treatment of developments in agriculture, such as genetic engineering, biological and chemical pest control, and the use of fertilizers and soil conservation measures, all of which may help food production to stay ahead of population growth. But there is a reluctance to discuss the more painful consequences for affluent countries of moves towards global equity. Much is made of measures such as investment in public transport systems, switching to vegetarian diets, and energy efficiency — the kind of adaptations for which it is easy to see health or even financial benefits in prosperous societies. 'Levelling down' is not seen as a viable option, and yet the consequences of rising affluence in China are so roundly condemned that one is left wondering how equity is to be achieved.

The dire consequences of economic growth in China are the subject of *Who Will Feed China?*, the latest in the World-watch Institute's Environmental Alert series, also aimed at a general readership. Using the historical relationships between industrialization and agriculture observed in Japan, South Korea and Taiwan (countries chosen because they too began to industrialize after achieving a high population density), Lester Brown predicts for China huge losses of prime agricultural land, growing divergence in living standards between rich and poor, a rise in demand for meat and dairy products, and increased dependence on food imports.

Various possibilities for agricultural intensification are analysed, such as increased irrigation, multi-cropping and advances in biotechnology. The conclusion, however, is that possible increases in domestic yields will not generate enough extra food to keep pace with rising demands because of natural constraints (such as aquifer depletion) or the rising cost of agricultural labour, which is seen as an inevitable consequence of industrialization. When China enters the international grain market as a major purchaser, Brown believes there will be food shortages on a global scale, because its size and purchasing power will consume all the surpluses generated in the exporting countries. He maintains that this would be disastrous for the poorest nations, especially those African countries now relying on food aid.

Will these warnings cause Chinese policy-makers to change direction and eschew rapid industrialization as the chosen path to development? Probably not. Although the problems are convincingly described, it is relatively easy to find logical errors in the unremittingly gloomy prognosis, and this will make it easier for its unwelcome messages to be ignored. Perhaps the biggest single flaw in Brown's arguments is that he does not allow for the possibility that China's development and

dietary trends may differ significantly from those of its predecessors precisely because of its size and the fact that it is a late entrant in the industrialization race. Significant rises in global food prices caused by increased demand for food in China could influence the relative value of agricultural and industrial land, and slow down or reverse the alienation of agricultural land. When China, with its relatively cheap labour supply, becomes a large-scale international supplier of manufactured goods, the pressure on wages in its industrial competitors could force a downward leveling of the more developed economies (as postulated by J. Cobb and H. Daly).

Both of these books describe historical progression from low-yield, labour-intensive agriculture to high-yield, mechanized

agriculture, heavily reliant on the chemical industry, and are concerned with the problems faced by the unemployed and the rural poor who lack the purchasing power or the land resources to obtain an adequate diet. Both are concerned with the effects of economic growth on the environment, but neither deals adequately with the complex range of feedback effects of environmental change on the economy, because neither has considered the possibility of the re-emergence of labour-intensive agriculture and industry as a response to shortages of natural resources. □

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On the wilder shores of cosmology

Michael Rowan-Robinson

A Short History of the Universe. By Joseph Silk. *Scientific American Library*: 1994. Pp. 246. £19.95, \$32.95 (hbk).

Perspectives in Astrophysical Cosmology. By Martin Rees. *Cambridge University Press*: 1995. Pp. 141. £24.95, \$39.95 (hbk); £9.95, \$19.95 (pbk).

The Nature of Space and Time. By Stephen Hawking and Roger Penrose. *Princeton University Press*: 1996. Pp. 141. \$29.95.

Einstein's Greatest Blunder? The Cosmological Constant and other Fudge Factors in the Physics of the Universe. By Donald Goldsmith. *Harvard University Press*: 1995. Pp. 216. £14.50, \$22.95.

Cosmology: A First Course. By Marc Lachièze. *Cambridge University Press*: 1995. Pp. 136. £25, \$49.95 (hbk); £13.95, \$19.95 (pbk).

COSMOLOGY is in a deliciously exciting state. The COBE satellite has demonstrated the reality of the 'hot Big Bang' and opened up speculation about the first 10^{-35} seconds of the Universe. The Hubble constant ought by now to have been determined but remains bafflingly uncertain. Dark matter appears to dominate much of the mass of the Universe but its nature remains unclear. It is scarcely surprising then that every year sees new books on cosmology at every level.

The present batch shows a very wide range in quality, scope and audience. The three books by eminent cosmologists, those by Silk, Rees, and Penrose and Hawking, are all, in their different ways, of high quality, and the serious student of cosmology needs to read all three. Before discussing them in detail, let me indicate my own prejudices by giving my assessment of the probability of some of the ideas that appear in these books being correct. I estimate the

probability that the hot Big Bang model gives a broadly correct description of the history of the Universe from 1 second after the Big Bang to the present as 99 per cent. To inflationary cosmology (the idea that very soon after the Big Bang the Universe went through a period of rapid exponential expansion), I assign a probability of 30 per cent. To string (or superstring) theory, the front-runner among theories to unify all the forces of nature and thereby quantize gravity, I give a probability of success of 10 per cent. And to Penrose's 'twistor cosmology' and Hartle and Hawking's 'no boundary proposal', I assign probabilities of only 1 per cent to their being the way forward. I say this only to emphasize that these ideas belong to the wilder shores of cosmology.

In Joseph Silk's *A Short History of the Universe* we encounter the more rewarding landscape of the origin of the stars, galaxies

and large-scale structures that we can actually observe. Silk gives a well-written and beautifully illustrated account of modern astrophysical cosmology, taking us through all the main questions. The basic concepts of the expansion of the Universe, the microwave background radiation, inflation, the origin of matter and the formation of helium, dark matter and its possible forms, and the origin of clusters and galaxies, are explained lucidly for the general reader. Because Silk wants to take us to the heart of the physical processes involved, this is a demanding book, but I believe it is the best introduction to cosmology for the general reader currently available.

Martin Rees's *Perspectives in Astrophysical Cosmology* is directed more at the beginning graduate student and gives an outline of what Rees sees as the important research questions in cosmology today. After introducing the cosmological framework, he turns his attention to galaxies and dark matter, the emergence of cosmic structure, quasars and their demography, probes and relics of the high redshift Universe, and finally to the early Universe and inflation. Although there are not many equations, the general reader would struggle because concepts such as the Planck time, the density parameter Ω and the Boltzmann factor are introduced without explanation. I liked Rees's remark that the evidence for Big Bang cosmology is at least as good as the evidence for the history of the Earth and also the dictum that he attributes to Landau that "cosmologists are often in error but never in doubt". This is a fascinating book which all cosmology graduate students and others with an interest in cosmology and some knowledge of physics should read. It is a mine of good PhD projects.

Stephen Hawking and Roger Penrose are well-known both for their work on the nature of black holes and for successful

HUBBLE Space Telescope image of the massive star Eta Carinae, surrounded by remnants of an outburst seen in 1843. Hubble has just discovered a unique natural ultraviolet laser in a nearby cloud. This picture appears in two new celebrations of the telescope's successes: *A Journey Through Time* (Penguin Studio, \$29.95) by Jay Barbree and Martin Caidin is gaudily written and, when coherent, astonishingly inaccurate; *Hubble Vision* (Cambridge University Press, £24.95, \$39.95) by Carolyn Collins Peterson and John C. Brandt succeeds modestly in combining a popular style with real astronomy. S.B.



books for the general public. *The Nature of Space and Time* has its origin in a series of lectures that they gave at a study programme in 1994 at the Isaac Newton Institute in Cambridge. They outline their past and current work on the global causal structure of space-time, singularity theorems, cosmic censorship, the laws of black-hole dynamics, quantum black holes, Schrödinger's cat paradox, the no-boundary proposal, the arrow of time, and the twistor view of space-time. This is an extremely demanding book, with many equations, and requires some knowledge of general relativity and quantum theory. The main theme of the lectures is the quantization of general relativity. Hawking's work on quantum effects near black holes and Penrose's on twistor theory have proved enormously illuminating, but few physicists, I think, believe that a full quantization of general relativity will be achieved along these paths. A better bet, surely, is string theory, despite the difficulty of developing this to the state of testable predictions. The claims made for the no-boundary proposal seem overstated. The 'prediction' of the microwave background fluctuations detected by COBE is a success also claimed by the whole generic class of inflationary theories. The debate between the positivist Hawking and the platonist Penrose on how the paradox of Schrödinger's cat should be understood is enjoyable, though the comparison with the classic debate between Einstein and Bohr in the 1920s on the correct interpretation of quantum theory, made by Michael Atiyah in his introduction, seems a bit grandiose.

Donald Goldschmidt previously wrote a good book on Supernova 1987A, but *Einstein's Greatest Blunder?* is less successful. It is a rambling and shallow account of the present controversies about the Hubble constant and the nature of dark matter in the Universe, and whether the introduction of the cosmological constant (essentially an additional repulsive force acting on large scales permitted in general relativity) can resolve them. I was irritated by the approach implied by the book's subtitle: *The Cosmological Constant and Other Fudge Factors in the Physics of the Universe*. There are some surprising omissions, for example the results from the surveys of galaxy redshifts undertaken by IRAS. In the list of underground dark matter experiments, the UK experiment, currently setting the best limits, is ignored. Some of the figures are crude and inaccurate. You will learn far more about these issues from Silk's book.

There are not a great number of introductory textbooks on cosmology for undergraduates, but Marc Lachièze-Rey's *Cosmology: A First Course* is not a very distinguished addition to the list. There is a poor and inaccurate introductory chapter with some astrophysical background but,

bizarrely, no mention of the microwave background radiation. The bulk of the book is a conventional account of the Friedmann models and their properties. The chapters on the early stages of the Big Bang and on galaxy formation give rather a limited physical insight. The greatest weakness is the virtual absence of discussion of observational cosmology. No index, references or list of further reading are provided.

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Neural mysteries

Alison Abbott

The Case of the Frozen Addicts. By J. William Langston and Jon Palfreman. *Pantheon*: 1995. Pp. 309. \$25.

"SWEDISH scientists use aborted fetuses to save drug addicts." This tabloid headline never existed, but the fear of it followed Bill Langston from the United States to Sweden where two of his patients underwent experimental surgery in 1989 to reverse their parkinsonism. This was a time when the world of neuroscience was gripped with excitement about a possible breakthrough in our understanding of Parkinson's disease. A chance discovery had opened up a new research direction in a field that for decades had stagnated, leading to an aggressive race to elaborate the biological mechanisms underlying the disease.

The discovery came not from a research bench but from the street, from the sad cases of six young Californian heroin addicts inadvertently exposed to a contaminated batch of a 'designer drug' in the summer of 1992. A few days after their bad shots, the addicts slowly froze. Completely unable to move or talk, the first victim, George Carillo, was presented to Langston, a local neurologist, who recognized the symptoms of severe parkinsonism, a degenerative motor disease normally confined to the elderly. Carillo had already been misdiagnosed by several doctors, all convinced he was malingering to avoid a prison sentence.

At the time, Langston was a clinical neurologist in a mundane county medical centre in California. He had ambitions to do basic research, but had neither the resources nor the training. The addicts proved to be his professional saving. *The Case of the Frozen Addicts* tells the tale of how Langston found his fortune in worlds initially foreign to him: the grim reality of drug addiction in America's underclass; the aggressive competitiveness of medical research; the devious politics of the

National Institutes of Health (NIH); and the ultra-conservative politics of the Bush administration. Langston, and the other bit-part actors in the story, had to confront all of these in solving the mystery of the frozen addicts and applying the knowledge to advance our understanding of Parkinson's disease and other disorders of voluntary movement.

It is a tale of several parallel strands. We learn how the police eventually tracked down the manufacturer of the contaminated drug (he was formerly a public-spirited lawyer); how sample analysis revealed the toxic contaminant to be MPTP; and how Langston and other scientists came to discover that MPTP, after metabolic conversion to MPP⁺, selectively destroys the dopamine-producing cells in the substantia nigra, a region of the brain involved in the control of movement. It was this finding that led to the first animal model of Parkinson's disease and so to an explosion of basic research into the disease.

The authors explain both the detective story and the science through the eyes of the personalities involved. One feels for Langston both when he suffers the practical consequences of professional jealousy and when he falls foul of the NIH, which 'poached' one of the addicts behind his back. (The moral here is that a small-time scientist cannot take on the might of the NIH.) Against all the odds, however, Langston managed to establish his own private treatment and research centre. We also gain insight into the minds of drug addicts, as well as sympathize with their agony, and that of their families, on finding themselves faced with a hellish choice: to remain trapped in an immobile body or to suffer the severe side-effects of the only medication, L-dopa, that could free their limbs.

The story has a happy ending. Three of the addicts have benefited from a controversial new technique, pioneered in Sweden, of transplantation of substantia nigra cells from human fetuses into the brains of parkinsonism sufferers. The use of fetal tissue in medicine was outlawed in the US just as the first scientists applied for permission to attempt such transplants at the NIH in 1988. (The ban was lifted in 1991.)

Although the authors provide an accurate and accessible description of the science, they do occasionally show a regrettable lack of judgement in giving equally enthusiastic weight to all theories, including the doubtful proposal that an environmental toxin, with a similar mechanism to MPTP, may be a prime cause of 'normal' parkinsonism. Nevertheless, the book succeeds in bringing science to life and showing its relevance to human health. It also illuminates the complexities that can surround researchers less isolated from the real world than they might otherwise imagine. □

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The 2.0 Å crystal structure of a heterotrimeric G protein

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The structure of a heterotrimeric G protein reveals the mechanism of the nucleotide-dependent engagement of the α and $\beta\gamma$ subunits that regulates their interaction with receptor and effector molecules. The interaction involves two distinct interfaces and dramatically alters the conformation of the α but not of the $\beta\gamma$ subunits. The location of the known sites for post-translational modification and receptor coupling suggest a plausible orientation with respect to the membrane surface and an activated heptahelical receptor.

MANY extracellular signals are mediated by heptahelical receptors coupled to heterotrimeric guanine-nucleotide-binding proteins (G protein; subunits, $\alpha\beta\gamma$)¹⁻³. In response to diverse stimuli, including light, hormones and neurotransmitters, heptahelical receptors activate heterotrimeric G proteins by catalysing the exchange of GTP for GDP bound to the G_α subunit. An agonist-stimulated receptor activates as many as several hundred G proteins, which in turn activate a variety of downstream effectors including enzymes, ion channels⁴, and intracellular signalling pathways such as the MAP kinase cascade^{5,6}. In the resting state, the GDP form of the G_α subunit ($G_\alpha\text{GDP}$) forms a high-affinity (nM) complex with the $G_{\beta\gamma}$ heterodimer. Binding of the heterotrimeric complex to an agonist-activated receptor results in the release of GDP. Subsequent GTP binding disrupts the complex with the receptor and leads to the dissociation of $G_\alpha\text{GTP}$ from $G_{\beta\gamma}$. Both activated $G_\alpha\text{GTP}$ and the released $G_{\beta\gamma}$ subunits are free to interact with downstream components of the signalling cascade. As a consequence of an intrinsic GTPase activity in the G_α subunit, GTP is hydrolysed to GDP, thereby returning the system to its heterotrimeric resting state.

One of the best characterized heterotrimeric G-protein-coupled pathways is the visual cascade of the rod cell^{7,8}, where the light-activated heptahelical receptor, rhodopsin, couples to the heterotrimeric G protein transducin ($G_{\alpha\beta\gamma}$). $G_{\alpha}\text{GTP}$ activates a potent cyclic GMP phosphodiesterase by displacing its inhibitory γ -subunits. A comparison of the structure of G_{α} complexed with either GDP⁹ or GTP- γS ¹⁰ (a non-hydrolysable GTP analogue) revealed the nucleotide-dependent structural changes that distinguish the GTP-bound (active) form from the GDP-bound (inactive) form. The structural changes occurring within the G_α subunit are not global, but rather are restricted to three adjacent 'switch' regions. Highly localized rearrangements within the context of an otherwise active GTP-bound conformation stabilize the transition state for GTP hydrolysis^{11,12}.

We now describe the refined 2.0 Å crystal structure of a heterotrimeric G-protein complex. A comparison with the previously determined $G_{\alpha}\text{GDP}$ structure⁹ and that of free $G_{\beta\gamma}$ (ref. 26) establishes the conformational changes that occur in each subunit to form the heterotrimer interface and hence reveals the mechanism of GTP-induced release and activation of $G_{\beta\gamma}$. The sites of post-translational modification on G_{α} and $G_{\beta\gamma}$ combined

with the receptor-binding regions of G_{α} , deduced from biochemical studies, occur on a common face that orients the heterotrimer with respect to the membrane surface.

Structure determination and refinement

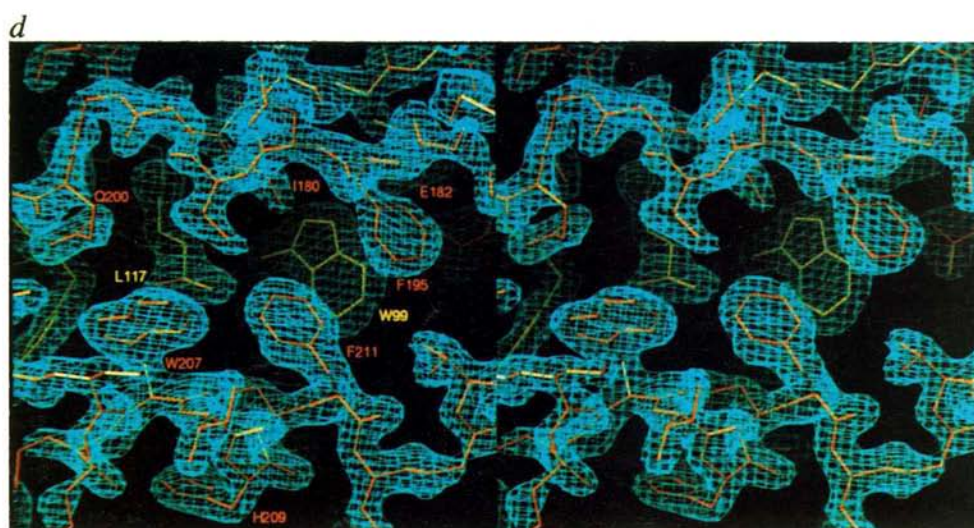
A modified heterotrimeric complex of transducin was prepared by mixing endo-Lys-C-proteolysed $G_{\beta\gamma}$ with a $G_{\alpha}/G_{\alpha 1}$ chimera ($G_{\alpha/1}$) in which residues 216–294 of G_{α} were replaced with the corresponding residues (220–298) from $G_{\alpha 1}$ (ref. 13). This chimera binds guanine nucleotides, assumes an active conformation in the presence of aluminium fluoride, and interacts with $G_{\beta\gamma}$ and rhodopsin in a light-dependent manner¹³. Proteolysis of $G_{\beta\gamma}$ with endo-Lys-C removes only the farnesyl modification and three residues from the C terminus of G_{β} while leaving G_{β} intact. $G_{\alpha/1}$ was expressed in *Escherichia coli* as a soluble fusion with an N-terminal 6xHis tag which was not removed before crystallization. Diffraction-quality crystals of the heterotrimeric complex in the monoclinic space group C2 were grown as described (Table 1).

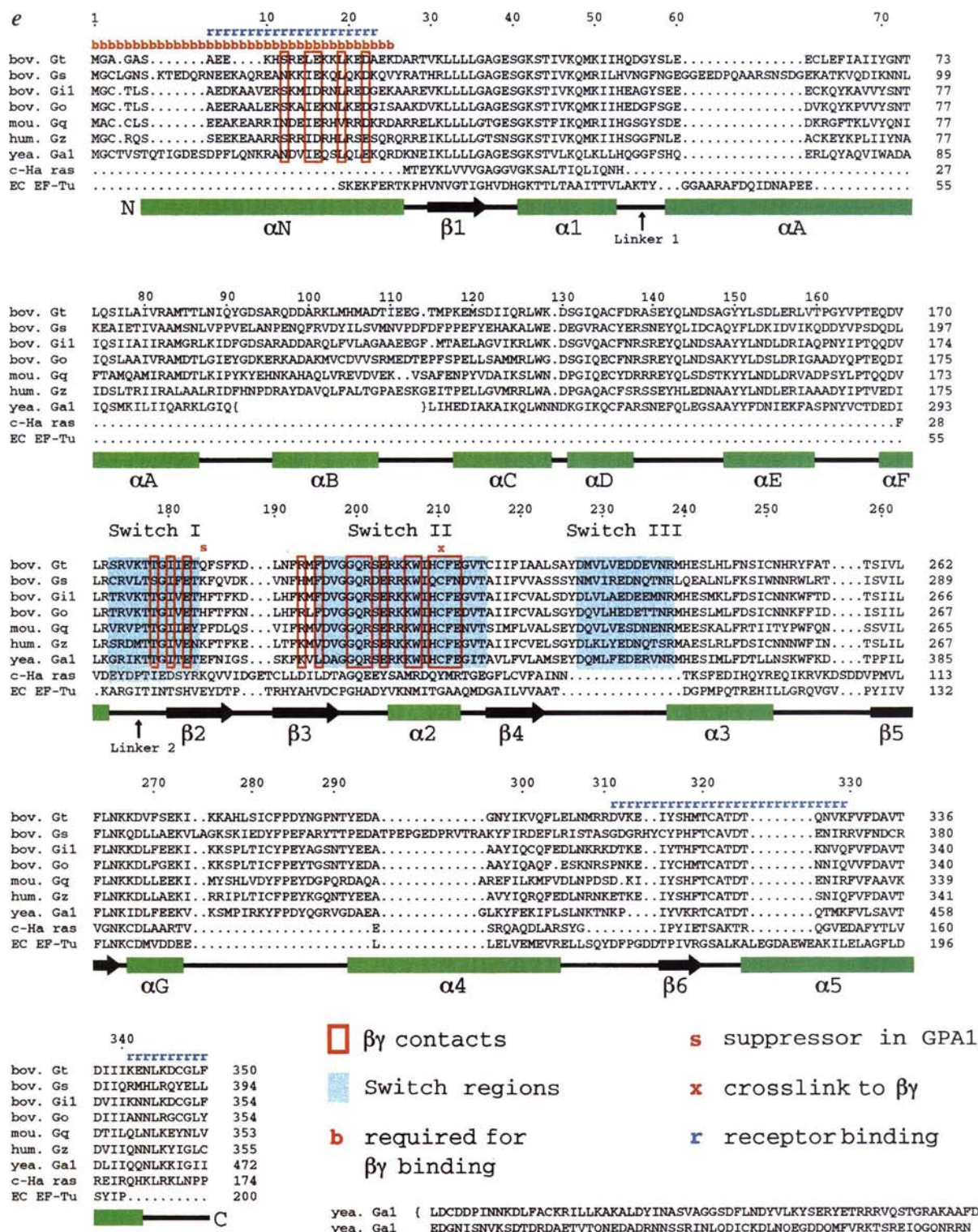
The structure was solved at 3.0 Å by multiple isomorphous replacement (MIR) using isomorphous differences from a Hg(OAc)₂ derivative and isomorphous as well as single-wavelength anomalous differences from a CH₃HgCl derivative and a selenomethionine (SeMet) derivative substituted in the $G_{\alpha/1}$ subunit. The initial map was improved with solvent flattening and histogram matching. A molecular replacement (MR) solution for the $G_{\alpha/1}$ subunit was obtained at 4 Å using the $G_{\alpha}\text{GDP}$ structure as a search model. Although the phases obtained from the MR solution were not sufficiently accurate to generate an interpretable map of the protein, they were useful for calculating derivative difference Fourier maps which allowed the location of heavy atom sites in each of the derivatives. Using Sigma A weights to reduce model bias, an interpretable map was calculated at 3.0 Å resolution with phases derived from multiple isomorphous replacement and anomalous scattering combined with phases from the partially refined molecular replacement model.

The model was refined with simulated annealing against the SeMet intensities from 6.0 to 2.0 Å resolution (Table 1). The present model includes residues 6–343 for $G_{\alpha/1}$, residues 2–340 for $G_{\beta\gamma}$, residues 8–66 for G_{β} , a GDP molecule, 630 water molecules, and has an *R* value of 20.7% for all data (19.6% for data $>2\sigma$) and a free *R* value of 29.5% (28.4% for data $>2\sigma$) for a randomly selected subset (10%) of the data omitted before the start of refinement. An example of the $2F_o - F_c$ electron density map is shown in Fig. 2d. The structure has

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excellent stereochemistry and ϕ - ψ angles within allowed regions of the Ramachandran plot. A more detailed description of the structure solution and refinement is given in the legend of Table 1.

Overall structure

The structure of the heterotrimeric $G_{\alpha\beta\gamma}$ GDP $\cdot$ $G_{\alpha\beta\gamma}$ complex is depicted in Fig. 1. The G_{α} subunit has three distinct structural components: (1) a Ras-like GTPase domain consisting of a six-stranded β -sheet surrounded by six helices ($\alpha 1$ – $\alpha 5$ and αG); (2) an entirely α -helical domain consisting of a long central helix (αA) surrounded by 5 shorter helices (αB – αF); and (3) an N-terminal helix (αN) that projects away from the remainder of the G_{α} subunit. The guanine nucleotide binds tightly in a deep cleft between the GTPase and helical domains. In general, the helical and GTPase domains as well as the guanine nucleotide closely resemble their counterparts in G_{α} GDP, except for significant changes where the GTPase domain contacts $G_{\beta\gamma}$. The N-terminal helix could not be observed in previously determined G_{α} structures^{9–11} where it was removed by proteolysis and was disordered in the structure of $G_{\alpha\beta\gamma}$ GTP γ S¹². The conformation of the N-terminal helix does not resemble that observed in the crystal structure of $G_{\alpha\beta\gamma}$ GDP¹⁴.

The $G_{\beta\gamma}$ subunit has an N-terminal helix followed by a repeating module of seven similar β -sheets, each with four antiparallel strands, that form the 'blades' of a β -propeller structure (Fig. 1a). The outside strand of each sheet, together with the inner three strands of the following sheet, comprise the structural unit corresponding to the 'WD'-sequence repeat first noted in $G_{\beta\gamma}$ ¹⁵. The G_{γ} subunit contains two helices and has no inherent tertiary structure. The N-terminal helix of G_{γ} forms a coiled-coil with the N-terminal helix of G_{β} whereas the remainder of G_{γ} interacts extensively with the β -propeller domain of G_{β} . The details of the $G_{\beta\gamma}$ structure are described in an accompanying paper²⁶.

G_{α} interactions with $G_{\beta\gamma}$

As shown in Fig. 2a, the interaction between the G_{α} and $G_{\beta\gamma}$ subunits occurs at two distinct interfaces. The most extensive interface involves residues in, or adjacent to, the switch I and II regions of G_{α} that interact with residues from loops and turns at the top of the β -propeller domain of $G_{\beta\gamma}$. The second interface is formed between the N-terminal helix of G_{α} and the side of the β -propeller domain of $G_{\beta\gamma}$. We shall refer to the former as the 'switch interface' because it involves regions of G_{α} that have been shown previously to undergo nucleotide dependent conformational changes and the latter as the 'N-terminal interface' because it involves only the N-terminal helix of G_{α} .

The switch interface buries $\sim 1,800 \text{ \AA}^2$ of solvent-accessible surface area and includes residues from $\beta 2$, $\beta 3$, and $\alpha 2$ in G_{α} as well as residues from five of the seven sheets in $G_{\beta\gamma}$. Fig. 2b shows the core of this tightly packed interface which consists of the side chains from six hydrophobic residues in G_{α} (Ile 180, Phe 195, Trp 207, His 209, Cys 210 and Phe 211) and four hydrophobic residues in $G_{\beta\gamma}$ (Tyr 59, Trp 99, Met 101 and Leu 117). The interaction is further stabilized by a network of hydrophilic interactions including both main-chain and side-chain hydrogen bonds and is flanked by two ion pairs. The specific interactions are shown schematically in Fig. 2g.

In contrast, only $\sim 900 \text{ \AA}^2$ of solvent-accessible surface area is buried in the N-terminal interface. As depicted in Fig. 2c, five residues on one side of the N-terminal helix of G_{α} (Ser 12, Glu 16, Leu 19, Asp 22 and Ala 23) interact with four residues from the first sheet of $G_{\beta\gamma}$ (Leu 55, Lys 78, Ile 80, Lys 89). The conserved Lys 89 plays an essential role through a tripartite interaction in which its backbone NH donates a hydrogen bond to the hydroxyl group of Ser 12 while its methylene groups pack against the side chains of Leu 15 and Leu 19 thereby positioning its amino group to form an ion pair with the carboxylate group of Glu 16. In addition to enhancing the stability of the complex, the N-terminal interface also determines the location of the modified N terminus of G_{γ} .

In contrast to extensive interactions with $G_{\beta\gamma}$, no direct interactions are observed between G_{α} and G_{γ} . However, the first five residues of G_{α} as well as the first eight and last four residues of G_{γ} are disordered. Therefore, the absence of interactions between G_{α} and G_{γ} in the present structure does not rule out the possibility that such interactions might occur under a more physiological setting. However, the extent of such an interaction would be limited.

Numerous biochemical and mutational studies have implicated the N terminus and switch II regions of G_{α} in the interaction with $G_{\beta\gamma}$. Limited proteolysis¹⁶, a monoclonal antibody directed against the N terminus^{17,18}, and expression of N-terminally truncated G_{α} subunits^{19,20} indicate that the myristoylated N terminus and some portion of the first 25 residues of G_{α} are essential for high-affinity binding to $G_{\beta\gamma}$. The ability of G_{α} to be ADP-ribosylated at Cys 351 by pertussis toxin is impaired by mutations at residues corresponding to Lys 17 and Lys 31 in G_{α} ²¹. Both residues are exposed to solvent and neither contacts $G_{\beta\gamma}$, suggesting that they may contribute to interactions with the toxin and/or the negatively charged membrane surface. The Gly 226 to alanine mutation in G_{α} that disrupts the GTP-induced conformational changes in the switch II region also blocks the release of $G_{\beta\gamma}$ ^{22,23}, consistent with the extensive interaction between $G_{\beta\gamma}$ and the switch regions of G_{α} . Finally, the conserved Cys 215 in the switch II region of G_{α} can be crosslinked by a 15 Å divalent alkylating agent to either of two conserved cysteines in $G_{\beta\gamma}$ (Cys 204 and Cys 271)²⁴. The crosslinks are compatible with the location of these cysteine residues in the structure (see Fig. 2e).

Regions of G_{β} that contact G_{α} have been implicated by yeast genetics. Mutational changes in the yeast mating factor G_{β} (ste4p) at residues corresponding to Trp 99 and Met 101 in G_{α} (Fig. 2e) cause constitutive activation of the pheromone signalling pathway and are defective for interaction with the yeast mating factor G_{α} (GPA1) in the yeast two-hybrid assay²⁵. Strikingly exposed on the surface of free $G_{\beta\gamma}$, these residues lie at the core of the hydrophobic switch interface with G_{α} in the heterotrimeric complex (Fig. 2b). In addition, some of the constitutively active mutants in the yeast G_{β} can be suppressed by a second site revertant (E307K)²⁵ at a residue corresponding to Gln 184 in G_{α} , which lies at the edge of the switch interface. Inspection of the heterotrimer structure predicts that a lysine residue at this position would be situated to form an ion pair with a non-conserved aspartic acid residue (Asp 133 in yeast G_{β}) located at the position of Arg 96 in $G_{\beta\gamma}$.

Most of the contacts involve residues that are highly conserved in both G_{α} (Fig. 1e) and G_{β} (see accompanying paper²⁶). Substitutions at these positions are typically conservative even in sequences from distantly related organisms. It is therefore likely that the interactions observed here can be generalized to other members the heterotrimeric G-protein family. Moreover, the structure does not reveal an obvious basis for specificity in the pairing of particular G_{α} and $G_{\beta\gamma}$ subunits. Thus, the extent to which specificity is determined at the level of the G_{α} interaction with $G_{\beta\gamma}$ or at the level of the heterotrimer interaction with the receptor remains an interesting question.

Conformational changes in G_{α}

Figure 3 shows a superposition of the previously determined structures of free G_{α} GDP⁹ and G_{α} GTP γ S¹⁰ onto that of G_{α} GDP in the heterotrimeric complex. The overall conformation is preserved except for residues within the switch I and II regions and a few flexible loops. The differences between the free and heterotrimeric forms of G_{α} GDP are most pronounced for residues that interact with $G_{\beta\gamma}$ directly. Some of these are critically conserved residues that are important to the structure and function of G_{α} GTP, such as Gly 199 which triggers conformational changes in the switch II region through an interaction with the γ phosphate in the GTP^{9,10}, and Gln 200 which stabilizes the transition state for GTP hydrolysis^{11,12}.

The most dramatic changes occur in the switch II region. In free

$G_{\alpha\beta}$ GDP, the $\beta 3$ strand terminates in a tight turn (Gly 198–Gln 200) that precedes the $\alpha 2$ helix (Arg 201 to His 209). As opposed to the taut and compact GTP-bound form, the $\alpha 2$ helix in free $G_{\alpha\beta}$ GDP is a loosely configured 3_{10} helix with conserved hydrophobic and polar residues exposed and accessible to proteases. In order to engage the $G_{\beta\gamma}$ subunit, the residues from Gly 198 to Ser 202 adopt an extended conformation, allowing the

remainder of the $\alpha 2$ helix to swing outwards, exposing and positioning residues to interact with $G_{\beta\gamma}$. As a consequence, the hydrogen-bonding network between the $\beta 2$ and $\beta 3$ strands is extended at the expense of that between $\beta 3$ and $\beta 1$. These changes do not significantly alter the solvent-accessibility of the nucleotide. However, it is likely that $G_{\beta\gamma}$ binding stabilizes the otherwise flexible switch I and II regions, perhaps accounting for the

TABLE 1 Structure determination and refinement

Crystal	Source	Data collection				R_{sym} (%)*		% Complete	
		Resolution limit (Å)							
Native	R-axis II	2.5				7.2		97	
SeMet	NLSL X25	2.1				6.1		99	
MeHgCl	R-axis II	3.5				9.1		85	
Hg(OAc) ₂	Xentronics	3.4				11.2		87	
MIR phasing statistics									
Resolution limit (Å)	16.90	10.17	7.27	5.66	4.63	3.92	3.40	3.00	Overall
Phasing power†‡ SeMet	0.32	0.63	0.54	0.53	0.38	0.28	0.23	0.23	0.30
SeMet (anomalous)	0.21	0.24	0.30	0.32	0.28	0.28	0.25	0.21	0.26
Hg(OAc) ₂	1.14	1.71	1.90	2.33	1.67	1.16	0.95	–	1.42
MeHgCl	1.42	1.58	1.79	2.41	1.93	1.34	0.95	–	1.55
MeHgCl (anomalous)	0.05	0.27	0.36	0.30	0.23	0.15	0.07	–	0.16
Mean figure of merit	0.43	0.57	0.56	0.54	0.46	0.36	0.23	0.13	0.31
Rotation search									
		Euler angles				Refined PC (Ω) §			
Search model	Θ ₁	Θ ₂		Θ ₃		Highest peak		Highest false peak	
G ₁₂ GDP	29.3	88.7		42.7		15.7σ		5.5σ	
Translation search									
		Fractional coordinates				Translation function %§			
Space group	x	y		z		Highest peak		Highest false peak	
C2	0.21	0.00		0.15		21σ		16σ	
Refinement									
		Resolution (Å)		R factor		Free R factor		No. of reflections	
Data with $F > 2\sigma$	6–2.0		19.6		28.4		49,438		
All data	6–2.0		20.7		29.5		54,174		
R.m.s. deviations	Bond lengths 0.01Å Bond angles 1.5°								

$G_{\alpha\beta\gamma}$ was isolated from photolysed bovine retinal rod outer segments (ROS) by selective extraction with GTP⁴⁹ and the subunits separated by chromatography on Cibracon blue Sepharose as described⁹. $G_{\beta\gamma}$ was proteolysed with 10 U of the lysine-specific protease endo-Lys C for 12 hours at 4 °C followed by cation exchange chromatography on Mono-Q (10 mM Tris, pH 7.5, 0.1–0.2 M NaCl gradient over 30 column volumes). The $G_{\alpha\beta\gamma}$ chimera was expressed as a soluble 6xHis fusion in *E. coli* as described¹³. Diffraction-quality crystals of the heterotrimeric complex were grown in microseeded hanging drops containing 10 mg ml^{−1} protein, 10% PEG-8000, 50 mM Tris, pH 8.0, 10% glycerol, 50 mM NaCl and 0.1% β-mercaptoethanol over a well containing the identical solution without the protein. Crystals appeared in 1–2 days and grew to a maximum of 0.3 × 0.3 × 0.5 mm in 1 week. The space group is C2 with unit cell dimensions of $a = 133.4$, $b = 91.4$, $c = 83.2$ Å. Crystals were incubated for 12 h at 4 °C in a cryoprotectant stabilizer solution containing 30% PEG-8000, 10% glycerol, 50 mM Tris, pH 8.0. The Hg²⁺ and CH₃Hg⁺ derivatives were obtained by soaking in the presence of 0.1 mM mercuric acetate and 1 mM CH₃HgCl respectively. Crystals were flash-frozen in liquid propane which was cooled with liquid nitrogen. The native and CH₃Hg⁺ derivative data sets were collected at −150 °C on an R axis II image plate system and processed with DENZO (Z. Otwinowski). The Hg²⁺ derivative data set was collected at 100K on a Xentronics area detector and processed with XDS⁵⁰. Diffraction data for the Se-methionine derivative were collected at 100K at the peak of the Se absorption edge at the X-25 beamline of the National Synchrotron Light Source at Brookhaven, processed with DENZO (Z. Otwinowski). All data sets were scaled with SCALEPACK (Z. Otwinowski). The location of the $G_{\alpha\beta\gamma}$ subunit was found by molecular replacement using as a search model the $G_{\alpha\beta}$ GDP structure in which residues in the range of the chimera were truncated to alanine. A rotation search using Patterson correlation refinement⁵¹ resulted in a single solution. A translation search yielded a clear solution with an R value of 50.5% after rigid-body refinement. Heavy-atom sites were located from isomorphous and anomalous differences Fourier phases calculated from the molecular replacement solution for the $G_{\alpha\beta}$ subunit. The coordinates and occupancies of the heavy atoms were refined with ML-PHARE. Initial MIR phases were improved by solvent flattening, histogram matching, and partial model combination using DPHASE (G. van Duyne). All computations for molecular replacement and subsequent refinement were carried out with X-PLOR 3.1 (ref. 52). Interactive model building was done with O (ref. 53).

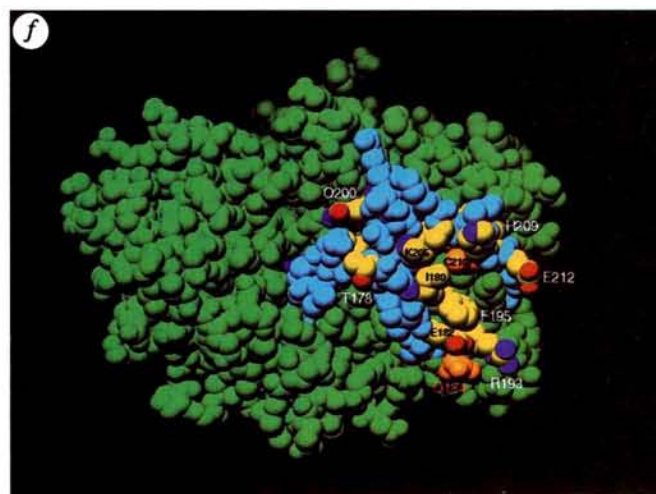
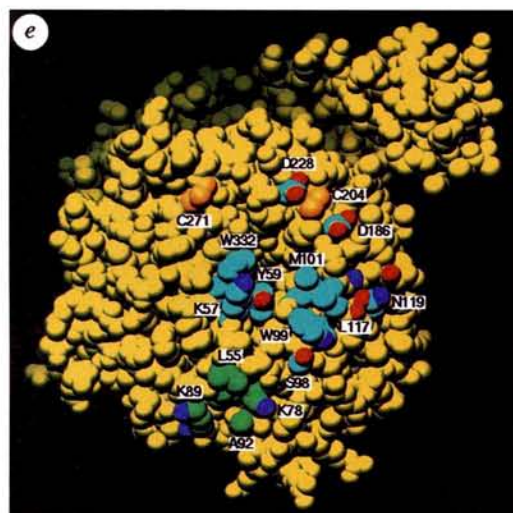
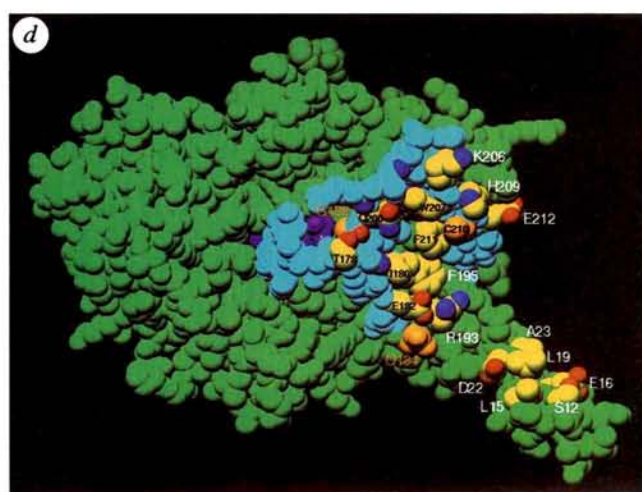
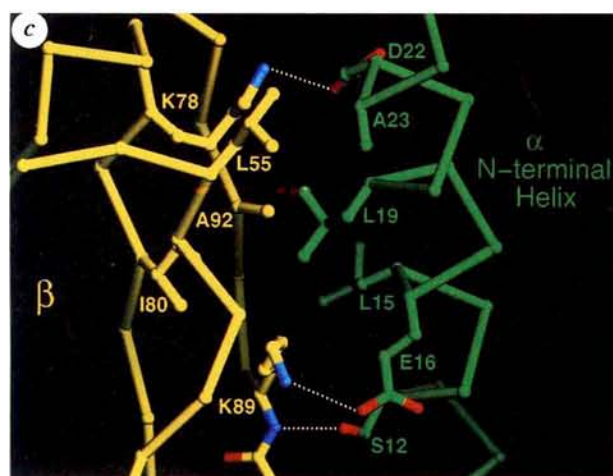
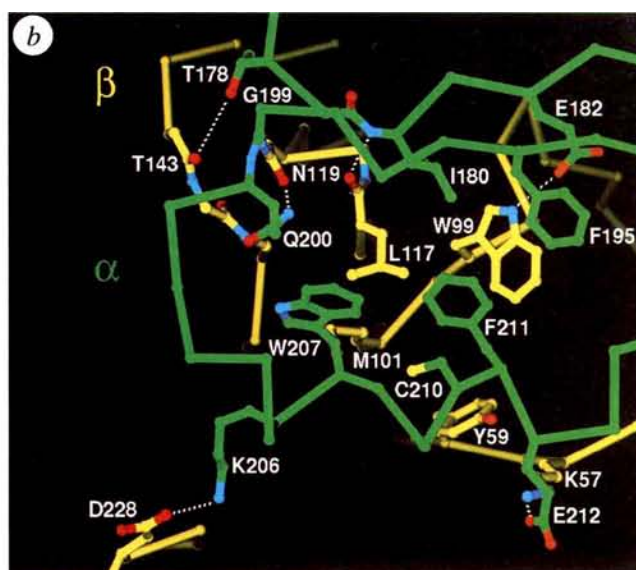
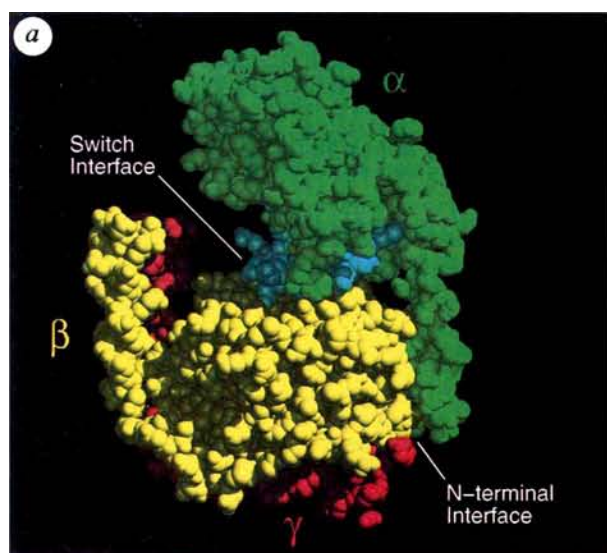
* $R_{\text{sym}} = \sum |I_h - \langle I_h \rangle| / \sum I_h$.

† Phasing power = $\sum |F_H| / \sum |F_{\text{PH obs}}| - |F_{\text{PH calc}}|$.

‡ Anomalous phasing power = $\sum |F_H''| / \sum |AD_{\text{obs}}| - |AD_{\text{calc}}|$.

$$\% \text{PC}(\Omega) = \frac{\langle |E_{\text{obs}}|^2 |E_m(\Omega)|^2 - \langle |E_{\text{obs}}|^2 \rangle \langle |E_m(\Omega)|^2 \rangle \rangle}{\left[\langle |E_{\text{obs}}|^4 - \langle |E_{\text{obs}}|^2 \rangle^2 \rangle \langle |E_m(\Omega)|^4 - \langle |E_m(\Omega)|^2 \rangle^2 \rangle \right]^{1/2}},$$

where E_{obs} denotes the normalized observed structure factors and E_m denotes the normalized structure factors for the search model placed in a triclinic unit cell with geometry identical to that of the crystal⁵³.



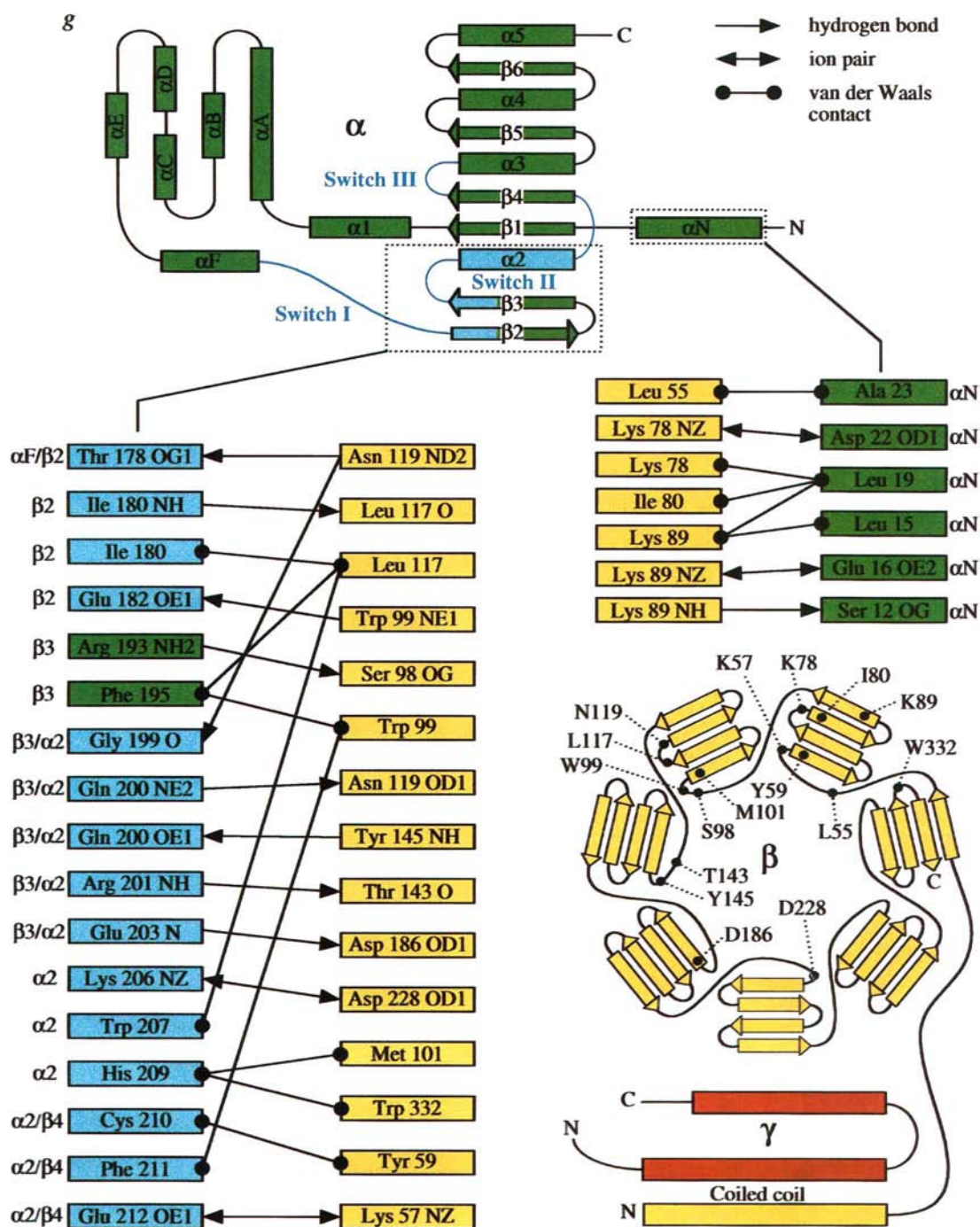
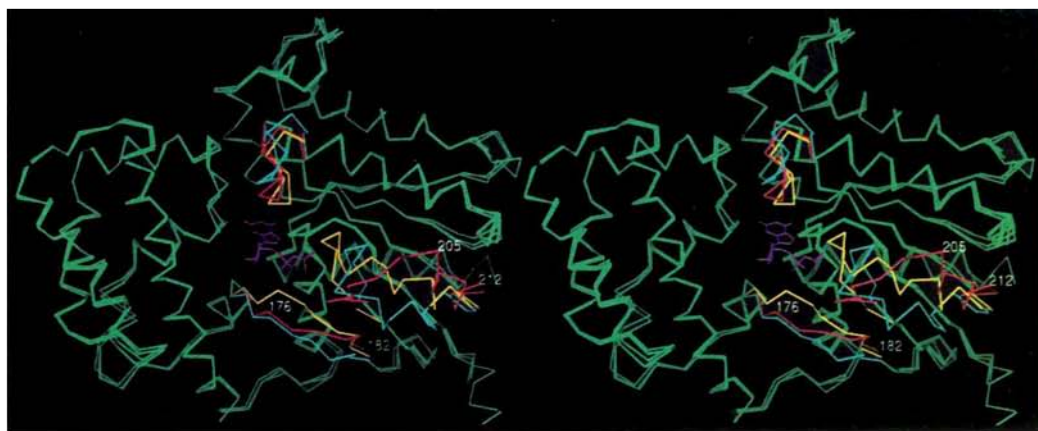


FIG. 2 Contacts in the two independent heterotrimer interfaces. *a*, Space-filling model showing the two distinct interfaces between the G_{α} and G_{β} subunits. *b*, Ball-and-stick model of the switch interface showing α -carbons (green in G_{α} ; yellow in G_{β}) and selected side chains (green in G_{α} and yellow in G_{β} , except for oxygens and nitrogens which are red and blue, respectively). *c*, Ball-and-stick model of the N-terminal interface showing α -carbons and selected side chains coloured as in *a*. *d*, Space-filling model of $G_{\alpha\beta}$ GDP (green) showing the switch I and II regions (cyan) and the residues that contact G_{β} (yellow, except oxygens and nitrogens which are red and blue, respectively). View is similar to the canonical orientation described previously⁹⁻¹¹. Residues identified in biochemical or mutational studies are highlighted in orange. *e*, Space-filling model of $G_{\alpha\beta}$ (yellow) showing the residues that contact $G_{\alpha\beta}$ GDP (green, except oxygens and nitrogens which are red and blue, respectively) in the switch interface. View is rotated by 180° about the y-axis compared to *d*. Residues corresponding to Trp 99 and

Met 101 have been implicated by yeast genetics²⁵ as essential for the interaction between G_{α} and G_{β} . Two cysteines in G_{β} (Cys 204 and Cys 271) that can be chemically crosslinked to Cys 215 in G_{α} (Cys 210 in G_{α})²⁴ are indicated in orange. Cys 210 lies at the edge of the switch II interface and makes van der Waals contact with Tyr 59 in G_{β} . At a distance of 13 Å from Cys 210, Cys 204 is located in a turn between the second and third strands of the fourth WD repeat and make van der Waals contact with the methylene groups of Lys 206 in G_{α} . Cys 271, on the other hand, occurs in the loop connecting the outer and inner strands of the sixth WD repeat and lies at a distance of 18 Å from Cys 210 and 10 Å from edge of the switch interface. *f*, Space-filling model of $G_{\alpha\beta}$ GTP·S (coloured and oriented as in *d*) showing how the switch interface is disrupted by the GTP-induced conformational changes. *g*, Diagram of the interactions between $G_{\alpha\beta}$ GDP and G_{β} in the heterotrimeric complex.

FIG. 3 Structural changes in G_{α} and $G_{\beta\gamma}$ subunits. Stereo pair showing the least-squares superimposed $C\alpha$ traces of free G_{α} GDP, heterotrimeric $G_{\alpha\beta\gamma}$ GDP, and G_{α} GTP γ S oriented as in Fig. 1a. All three molecules are coloured green except for the switch I and II regions, which are blue for free G_{α} GDP, red for heterotrimeric $G_{\alpha\beta\gamma}$ GDP, and yellow for G_{α} GTP γ S.



observation that the interaction with $G_{\beta\gamma}$ decreases the rate of GDP release²⁷. On replacement of GDP with GTP, local but dramatic conformational changes disrupt nearly all of the contacts in the switch interface, thereby triggering the disengagement of G_{α} and $G_{\beta\gamma}$. It is also likely that the structure of the N-terminal helix of G_{α} depends on the interaction with G_{β} , as its conformation in the heterotrimer differs markedly from that observed in the G_{α} GDP structure¹⁴.

Implications

In contrast to the dramatic structural reorganization in the switch I and II regions of G_{α} , significant structural changes are not observed in $G_{\beta\gamma}$. Although the structures of $G_{\beta\gamma}$ alone and bound to G_{α} were independently determined, the r.m.s. deviation in $C\alpha$ positions after superposition is only 0.72 Å. Thus, the $G_{\beta\gamma}$ subunits appear to function as a rigid unit with critical residues prepositioned to interact with G_{α} GDP or other signalling components. Whereas G_{α} activation proceeds through nucleotide-dependent structural reorganization, activation of $G_{\beta\gamma}$ occurs solely as a consequence of its release. This implies that the G_{α} subunit acts as a negative regulator of $G_{\beta\gamma}$ by restricting its degrees of freedom and/or masking sites on the surface of G_{β} that interact with downstream signalling components. The regions of yeast $G_{\beta\gamma}$ that interact with downstream signalling components have been mapped genetically^{54,57}. None of the residues implicated in these studies lie within either of the two interfaces between G_{α} and $G_{\beta\gamma}$. Instead, they map to the coiled-coil and a region immediately adjacent to the switch interface (Fig. 3 of accompanying paper²⁶). However, as both G_{α} and effectors are large macromolecules, steric constraints will extend the masked region of G_{β} beyond the actual van der Waals contact surface, thereby extending the effective 'footprint' of G_{α} on $G_{\beta\gamma}$. Thus the G_{α} and effector binding sites on $G_{\beta\gamma}$ need not overlap directly. In this respect, the helical domain of G_{α} (Fig. 1b) appears to be positioned such that it could sterically hinder the access of effectors that bind to regions similar to those implicated for yeast $G_{\beta\gamma}$.

Both the G_{α} and $G_{\beta\gamma}$ subunits undergo post-translational modification, resulting in the covalent attachment of specific lipophilic groups. The G_{γ} subunits are farnesylated ($G_{\gamma 1}$)^{28,29} or geranyl geranylated ($G_{\gamma 2-4}$)³⁰⁻³² at the cysteine in the C-terminal CAAX motif. Several G_{α} subunits are subject to N-terminal myristoylation ($G_{\alpha 1}$, $G_{\alpha 2}$, $G_{\alpha 3}$)³³ or heterogeneous acylation ($G_{\alpha 4}$)^{34,35} at Gly 2 and most (except $G_{\alpha 1}$) are regulated by reversible palmitoylation at Cys 3^{36,37}. These modifications are essential for membrane association. In addition, myristoylation of G_{α} ³⁸ and prenylation of G_{γ} increase the stability of the heterotrimer complex³⁹. Experiments with G_{α} indicate that this stabilization occurs only in the presence of membranes or detergent micelles, suggesting that the primary role of the lipophilic modifications is to co-localize and orient the subunits on the membrane surface⁴⁰.

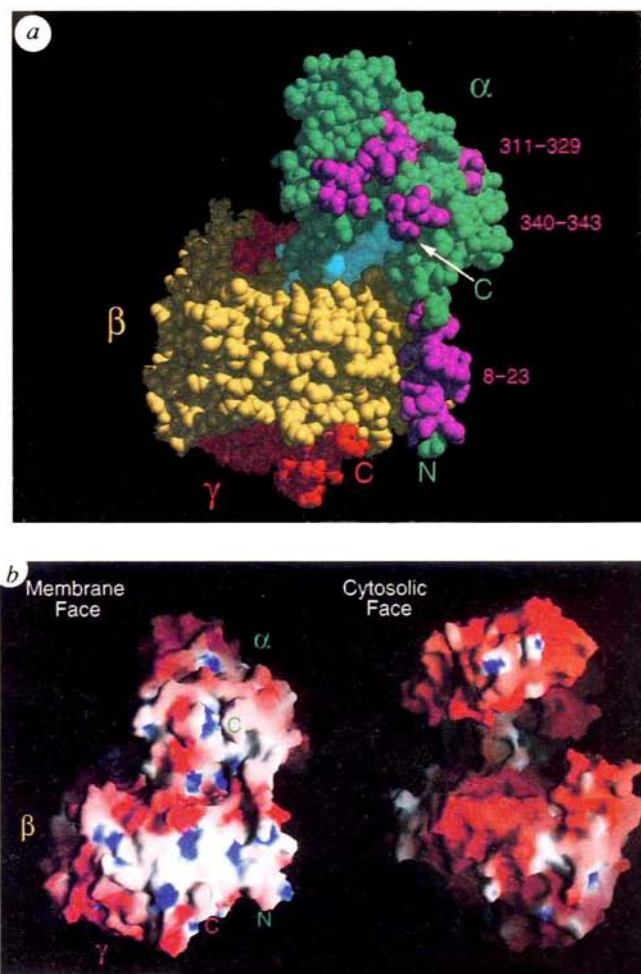


FIG. 4 Face of the heterotrimeric complex that is proposed to interact with the membrane surface and photoactivated rhodopsin. *a*, Space-filling model highlighting the sites of hydrophobic post-translational modification (N terminus of G_{α} and C terminus of G_{γ}) and regions that are known from biochemical experiments to contact receptors (purple). The orientation is rotated about the vertical axis by $\sim 70^\circ$ with respect to the view in Fig. 1b. *b*, Solvent-accessible surface coloured according to electrostatic potential using GRASP⁵⁶. The electrostatic potential is contoured in the range from $-10 k_B T$ (red) to $+10 k_B T$ (blue) where k_B is Boltzmann's constant and T is the absolute temperature (K).

Although the G_x and G_y subunits in this study lack these modifications, their sites of attachment can be approximated from the location of the unmodified N- and C-termini of G_x and G_y , respectively. In the present structure, the first four residues (Gly-Ala-Gly-Ala) from the N terminus of G_x and the last four residues (Lys-Gly-Gly-Cys) from the C terminus of G_y are not observed. The amino-acid sequences suggest that both regions could be conformationally flexible in the absence of other interactions. Moreover, the structures of G_{tx} and G_{ty} do not reveal obvious binding sites for large hydrophobic groups. Significantly, the observed N terminus of G_{tx} and the observed C terminus of G_{ty} are located 18 Å apart on a common face of the heterotrimer. Thus, the structure is consistent with the hydrophobic modifications inserting simultaneously and cooperatively into the lipid bilayer rather than binding to sites on the protein and also supports the conclusion that the enhanced stability of the heterotrimer complex results from co-localization of the subunits to membranes or detergent micelles.

The receptor binding surface of G_x has been mapped approximately by biochemical and mutational data. Many experiments indicate that the C terminus of G_x is an essential region for receptor coupling. ADP ribosylation by pertussis toxin⁴¹, mutations^{42,43} and peptide-specific antibodies⁴⁴, all directed at the C terminus, uncouple G proteins from receptors. Experiments with G_x chimaeras indicate that this region is also important in determining G-protein receptor specificity⁴⁵. A peptide corresponding to the C-terminal 11 amino acids has been shown to interact directly with photoactivated rhodopsin^{46,47}. In addition, the C-terminal peptide and peptides corresponding to the end of the $\alpha 4/\beta 6$ loop through the beginning of $\alpha 5$ (residues 311–329) as well as most of the N-terminal helix (residues 8–23) inhibit G_t binding to rhodopsin⁴⁷.

The approximate locations of the sites of post-translational modification and receptor coupling occur on a common face of the heterotrimeric complex (Fig. 4a). This side of G_{ty} is surprisingly flat, and electrostatic calculations reflect a predominantly

neutral surface interspersed with patches of positive charge, in contrast to the other faces where the electrostatic potential is dominated by negatively charged residues (Fig. 4b). Thus, a plausible model would orient this face of the heterotrimer against the negatively charged membrane surface allowing the hydrophobic modifications to insert simultaneously into the lipid bilayer while positioning the known receptor contact sites of G_x to interact with an activated heptahelical receptor. In addition, G_{ty} has also been shown to interact directly with rhodopsin⁴⁸; however, the regions that contact the receptor have not been identified. One possible receptor contact site on G_{ty} , consistent with the location of the approximate receptor contact regions of G_x (Fig. 4a), is the region adjacent to the switch and N-terminal interfaces. The observation that G_{ty} interacts with both the receptor and the switch I/switch II region of G_x , which flanks the nucleotide binding site and buttresses the critical phosphate binding loop, gives stereochemical support to a potential role for G_{ty} in receptor-catalysed nucleotide exchange. In addition, the presence of receptor contact sites on both G_x and G_{ty} makes the stability of the receptor–G protein complex dependence dependent on the interaction between the subunits, and hence provides a simple mechanism for coupling the GTP-triggered dissociation of G_x and G_{ty} to their release from the receptor.

Ultimately, a mechanistic understanding of the nucleotide-exchange process will require the structure of an activated heptahelical receptor bound to the nucleotide free form of a heterotrimeric G protein. In the interim, the present study provides the basis for mutational experiments designed to test specific hypotheses regarding the mechanism of nucleotide exchange and better define the role of G_{ty} in facilitating this process.

Note added in proof: After this paper was submitted, Wall *et al.* (*Cell* **83**, 1047–1058, 1995) published a partially refined structure of $G_{ix1\beta 1\gamma 2}$ showing a similar overall architecture. However, a comparison of the intersubunit contacts of the G_t heterotrimer with those of the G_i heterotrimer is compromised by an admitted lack of 'atomicity' in the $G_{ix1\beta 1\gamma 2}$ model. □

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Kilometre-scale structures in the Sun's corona

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KNOWLEDGE of the structure of the Sun's corona is important for our understanding of how this high-temperature plasma is heated, and of the processes involved in the acceleration of the solar wind^{1,2}. The structure can be investigated directly by imaging at optical and shorter wavelengths, or indirectly through the effects of changing electron density on the propagation of radio waves (scattering and scintillation). Radio measurements have established many of the characteristics of the density fluctuations in the corona and solar wind, but the fundamental nature of these structures is not yet fully understood^{3,4}. Two specific features that have proved difficult to explain are an abrupt increase in anisotropy of the irregularities close to the Sun⁵⁻⁷, and a break in the power-law spectrum describing the density fluctuations^{8,9}. Here I argue that these features are the manifestation of a transition from small ray-like or filamentary structures in the corona that rotate with the Sun to turbulent density irregularities convecting with the solar wind. I estimate the size of the smallest filamentary structure within coronal holes to be about 1 km at the Sun, approximately three orders of magnitude smaller than the smallest filamentary structures observed in images of different wavelengths^{2,10-12}.

Filamentary structures have been discussed since the earliest investigations of the solar corona¹³⁻¹⁵. There is growing evidence that these structures are ubiquitous in the solar corona and that they extend to ever-smaller scales, with detection seemingly limited only by the spatial resolution of the measurement^{2,10-12}. Although electron-density fluctuations observed by radio scattering and scintillation measurements have traditionally been interpreted in terms of electron-density irregularities that are convected along with the solar wind⁹, recent results based on Doppler scintillation measurements of a coronal streamer have not only shown that the fluctuations can also represent filamentary structures, but that they provide evidence for the extension of these structures to sub-arcsecond scales¹⁶. Clearly, a transition must take place in the corona, from filamentary structures to the convected electron density irregularities that have been extensively observed in intensity scintillation measurements^{4,17,18}. This transition is illustrated in Fig. 1, which is a schematic snapshot of the corona. The corona consists of filamentary structures (represented by circularly symmetric flux tubes aligned along the magnetic field) and convected electron density irregularities within them.

The break near 1 Hz in the inverse power-law density spectrum inferred from phase scintillation and spectral broadening measurements^{8,9} marks the separation between the Kolmogorov spectrum (spectral index of 5/3) observed at lower fluctuation frequencies and the flatter spectrum (spectral index of about one) at higher frequencies, and suggests a change in the nature of the irregularities. In the model proposed here, the break in the density spectrum corresponds to the boundary of the flux tube, so that inside the flux tube the flatter spectrum represents the density variations across the small-scale irregularities in directions both parallel and transverse to the flux tube (magnetic field). Outside the flux tubes, the Kolmogorov spectrum represents the density variations across the filamentary structures. The Kolmogorov spectrum has been found in some theoretical models of the solar wind¹⁹⁻²², and is used in models for heating the corona, the chromosphere and active region loops²³⁻²⁵.

The sizes of the co-rotating flux tubes can be estimated from measurements of the break in the density spectrum, as size is related to the break frequency by the Sun's rotation rate (once every 27 days). Shown in Fig. 2 are the estimates obtained at 5, 10, and 20 $R_{\odot}$ based on the density spectra reported in ref. 8. These spectra were observed in the absence of enhanced density fluctuations²⁶, so they are representative of equatorial coronal hole rather than coronal streamer regions.

The high anisotropies observed in interferometric angular broadening measurements can be explained in terms of the underlying density spectra. When the interferometer baselines are short enough, or when the measurements take place far enough from the Sun that the observations sense scales inside the flux tubes, then the angular scattering measurements are nearly isotropic, reflecting the near-isotropic irregularities within the flux tubes. These observational conditions prevailed during many of the earlier angular broadening measurements²⁷.

When the interferometer baselines are long enough, or when the measurements take place close enough to the Sun that the observed irregularity scales exceed the flux tube size—as has been the case in measurements by the National Radio Astronomy Observatory's Very Large Array (VLA)—high anisotropies are observed⁵⁻⁷. This stems from a significant increase in the scattering in the transverse direction relative to that in the radial direction, owing to the steepening of the density spectrum beyond the flux tube size. The onset of high anisotropy in this view signals the crossing of the flux tube, and an approximate measurement of the flux tube size is given by the interferometer baseline in the direction transverse to the flux tube when the high anisotropy is detected. VLA measurements of a polar coronal hole region were carried out in 1985 with a maximum baseline of 35 km (ref. 6). Shown in Fig. 2 are the sizes of the flux tubes estimated from the interferometer baseline in the transverse direction (by dividing 35 km by the anisotropy) when an anisotropy of five or larger was observed.

The flux tube sizes in Fig. 2 show that the smallest filamentary structure, like the much larger ray-like structures such as polar coronal plumes and coronal streamers^{12,16,28}, expands approximately radially. When extrapolated to the Sun, the inferred flux tube size is about 1 km ($\sim 10^{-3}$ arcsec), and corresponds to a longitudinal or latitudinal extent of 0.3" in heliographic coordinates. The remarkable order and consistency of the results in Fig. 2 demonstrate that the picture of striated plasma co-rotating with the Sun answers long-standing questions about the angular broadening and phase/Doppler scintillation measurements, both individually and collectively. It is especially significant that the collective agreement is a consequence not of relating the spatial (angular broadening) and temporal (phase/Doppler scintillation) variations through the solar-wind speed, as would be the case for convected turbulence, but of relating them through the Sun's rotation rate. The combined results therefore provide evidence for the rotation of the very small structures with the Sun and its magnetic field. Although the filamentary structures rotate in a

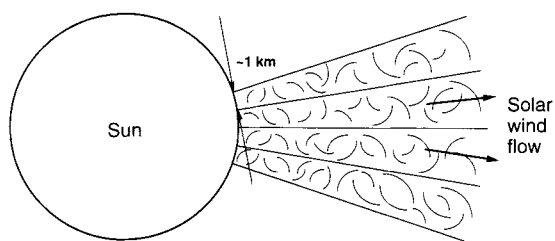


FIG. 1 Schematic snapshot of the corona showing the smallest filamentary structures, represented by circularly symmetric flux tubes aligned along the magnetic field with convected electron density irregularities inside them.

direction generally parallel to the radio path, for reasons such as the small size of the flux tubes, the tilt in the Sun's rotation axis relative to the ecliptic plane, and departures of the structures from radial alignment, enough of them cut across the radio path at the Sun's rotation rate to dominate the path-integrated measurements and to produce the distinct break in the observed density spectrum.

Angular broadening measurements play a unique role among radio scattering measurements. They are the only measurements that image the electron density irregularities (structures) in the plane of the sky, in directions both parallel and perpendicular to the magnetic field. Furthermore, they yield direct and robust measurements of the spatial spectrum, without any assumptions about the velocity of the scatterers. So far, the break in the transverse density spectrum has not been directly inferred from the angular scattering measurements themselves, but only indirectly through the onset of anisotropy discussed above. However, comparison of measurements from the VLA and the VLBA (Very-Long-Baseline Array) hints at the existence of this break. Whereas the inverse power-law spectra for the smaller-scale density irregularities obtained from the VLA (< 35 km) show⁶ a spectral index near one, those for the larger-scale irregularities (> 200 km) from the longer-baseline VLBA (for which parallel and transverse directions are not distinguished) gives²⁹ a spectral index closer to the Kolmogorov case of $5/3$. It is clear that interferometric measurements of the density spectra in the parallel and transverse directions are invaluable. Of particular interest are the break in the spectrum in the transverse direction, and the convected irregularities in the radial direction with scales that are larger than the flux tube size, which cannot be observed by any other radio measurement. Angular broadening measurements should also lead to a better understanding of the large-scale structures and their relationship to those observed in white light measurements, as has recently been demonstrated with Doppler scintillation measurements¹⁶.

The spatial structures in the corona have manifestations in the solar wind. They appear to evolve quickly with increasing heliocentric distance in the fast wind, presumably because of lateral interaction of the flux tubes, as evidenced by the growth in the density fluctuations²⁶ and the shift of the break in density spectrum to lower fluctuating frequencies observed by both scintillation and *in situ* measurements^{30,31}. By 0.4 AU, the break in density spectrum has moved to about 3×10^{-4} Hz (a period of ~ 1 h), and if this break still represents the smallest flux tube, then the indication is that it has grown to an angular scale of 0.5° , consistent with the *in situ* observations of such structures in radially aligned measurements³².

There is no incompatibility in rotation and convection as a source of plasma motion in the case of spaced-receiver measurements based on intensity scintillation^{4,17,18}, because the nearly isotropic electron density irregularities³³ that give rise to intensity scintillation are on spatial scales smaller than the Fresnel size, and hence are almost always smaller than the flux tube size. The phase scintillation and Faraday rotation fluctuations lower than ~ 1 Hz caused by co-rotating structures (as well as large-scale irregularities convected along with the solar wind) may be one of the reasons why intercontinental-baseline multiple-station phase scintillation measurements do not always behave as expected³⁴ and why multiple-station Faraday rotation measurements sometimes yield negative flow velocities³⁵.

Taken together, images of the corona and recent results from radio scattering measurements suggest that the corona is permeated by filamentary structures, starting from large-scale structures such as polar plumes and coronal streamers, and extending down to scales as small as 1 km at the Sun. The consistency between measurements in the spatial and temporal domains provides compelling evidence for the rotation of the smallest filamentary structures with the Sun. Because plumes and streamers are aligned with the solar magnetic field and rotate with the Sun, and larger-scale structures appear to be composed of

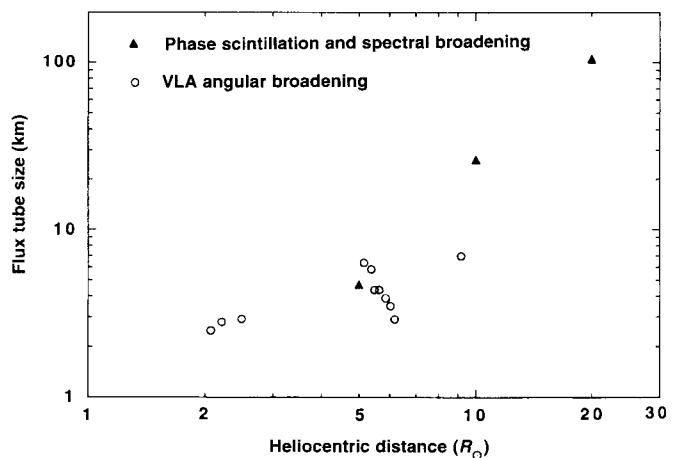


FIG. 2 Flux tube size as determined from the breaks in the density spectra inferred from phase scintillation and spectral broadening measurements of ref. 8 (filled triangles), and from the transverse baselines deduced from the VLA angular broadening measurements of ref. 6 (circles).

smaller filamentary structures, it seems likely and reasonable that most of the structures, whose variations are characterized by the Kolmogorov spectrum, should also rotate with the Sun. Although angular broadening measurements can provide further clues, it is not possible to unambiguously determine whether all structures rotate with the Sun. Thus, the large-scale variations could represent dynamic two-dimensional turbulence or discrete structures ejected from the Sun³⁶. Whether quasi-static or dynamic, it is clear that radio scattering measurements of the solar corona can provide important insight into the role of structures² and turbulence²³ in the heating of the corona and the acceleration of the solar wind. □

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Rapid energy dissipation and variability of the Io–Jupiter electrodynamic circuit

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THE electrodynamic interaction between Jupiter and the closest of its large moons, Io, is unique in the Solar system. Io's volcanoes eject a considerable amount of material into the inner jovian system (>1 tonne per second), much of it in the form of ions¹; the motion of Io through Jupiter's powerful magnetic field in turn generates a million-ampere current² between the charged near-Io environment and the planet's ionosphere. This current is presumably carried by Alfvén waves³, the electromagnetic equivalent of sound waves. Here we present far-ultraviolet observations of the atmospheric footprint of this current, which demonstrate that most of the energy is dissipated rapidly when the waves first encounter Jupiter's ionosphere; the position of the footprint varies with time. We see no evidence for the multiple ionospheric interactions that have been proposed to explain the structure of the radio emissions associated with these waves⁴.

The images we present here were taken in the far-ultraviolet (FUV) using the Faint Object Camera aboard the Hubble Space Telescope (HST), after the successful COSTAR upgrade. This upgrade enabled us to pinpoint, for the first time in the FUV, the specific footprint of the moon Io, well resolved from, and as bright as, the auroral emission. The FUV emission is caused by collisional excitation, a uniquely direct signature of a particle-precipitation process. Thus we have been able to detect jovian aurora directly generated by Io. Our observations also allow us to use a relationship between auroral brightness and particle-precipitation flux to estimate primary energy deposition⁵.

Previously, long-term observations of jovian radio emissions and Voyager data had suggested an electrodynamic circuit closing through Io and Jupiter's ionosphere, with a system of intense magnetic-field-aligned currents, generally believed to be carried by kinetic Alfvén waves generated by Io's motion across the jovian magnetic field^{6–9}. Several important issues were raised by these earlier observations. (1) To what extent the source of the current system is localized to the immediate vicinity of Io, rather than in an extended Io wake or Io magnetosphere (as discussed by Southwood *et al.*⁹ when considering the *in situ* data from Voyager). (2) If the source were Io, to what extent, if any, the path of the current deviates from the instantaneous field tube (IFT) connecting Io to the jovian ionosphere; the jovian radio bursts indirectly suggest^{10,11} active field lines ahead of it ('leading') by 20° on average with a dispersion of $\pm 15^\circ$, whereas present-day Alfvén-wave interaction models can account only for a few degrees. (3) Whether the energy is deposited directly, or after a number of interhemispheric reflections on the ionosphere or on the dense Io plasma-torus, as suggested by the 'multiple-arc' structure found in radio emissions from Voyager and ground-based observations^{4,12}.

Connerney and co-workers¹³ have recently identified the IFT

footprint from ground-based observations in the near-infrared H₃⁺ rotational-vibrational transitions. The infrared hotspot lies some 15–20° ahead of surface footprint of the IFT.

Figure 1 shows the north (top of image) and south (bottom) polar regions of Jupiter on 9 August, 1994, in the H₂ Lyman bands near 1,550 Å. The physical pixel size is (0.014 arcsec)². The total energy point-spread function (PSF) at half-maximum is <0.05'' in radius (3 pixels), including contributions of the telescope, camera and filters, exceeding by far the capabilities of any other instrument.

The important feature in Fig. 1 is the bright spot in the southern hemisphere near the east limb (left on the figure), north of the auroral oval. At the time, Io was on the dayside, near the dawn meridian, and there is every reason to identify the spot with the locus of energetic-particle precipitation from a source connected in some way to Io.

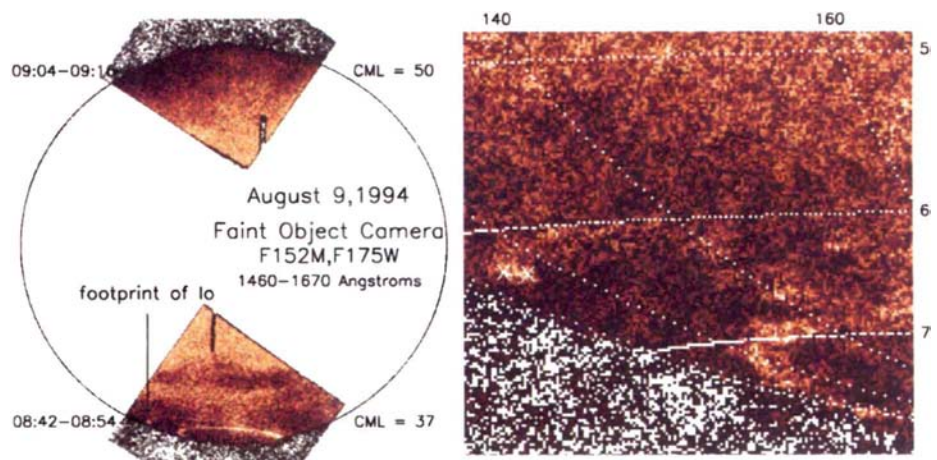
Figure 3 shows photon count-rates along tracks across the spot. The spot is narrow in latitude (about the size of the PSF) and slightly elongated in longitude. The apparent longitudinal extent, $\sim 5^\circ$ at half-maximum, exceeds only slightly the distance the Io footprint moved during exposure. This is consistent with the light being emitted from an instantaneous source comparable in size to Io's projection along the magnetic field. The bulk of the interaction must be confined within a few radii of Io, with Io itself or its ionosphere; much less, if any, seems to originate from a cometary-like Io wake or from a reconnected Io magnetosphere possibly extended along Io's orbit (Fig. 1).

The total power radiated by the spot in the H₂ Werner–Lyman bands is $\sim 5 \times 10^{10}$ W. Using a model of energy degradation for the precipitating particles⁵, we can deduce the fraction of the incident energy going into excitation of H₂, and we estimate the power input from precipitation in the IFT as $\sim (2-3) \times 10^{11}$ W. This is a remarkably high figure, showing that the energetic electrons deposit $\sim 1/4$ of the total system energy provided by the motion of Io across jovian magnetic field lines (estimated⁸ as of the order of 10^{12} W per hemisphere) in this one spot. As there must also be substantial, comparable energy dissipated by Joule heating of the atmosphere, it seems that most of the energy of the Alfvén waves, which carry the disturbance, is deposited in the first interaction with the ionosphere. It is thus unsurprising that our images show evidence only of a single bright spot. But this raises a serious problem. 'Multiple-arc' patterns are consistently observed in Io-controlled frequency-time spectrograms of the decametric radio emission^{12,14}. The most satisfying explanation proposed multiple bounces of standing Alfvén waves between the northern and southern ionospheres, or between the ionosphere and the dense plasma-torus generated by Io along its orbit^{4,7,15}. Longitudinal spacings of a few degrees are expected between adjacent Alfvén wings ($\sim 5^\circ$ on average) and up to 100 consecutive bounces could occur before the wave is totally absorbed. This implies good reflection on the ionosphere, and only a small energy loss on each bounce, less than 10–20% (refs 4, 16). This does not seem to fit with our data which support large local energy dissipation in the first ionospheric interaction ($\geq 50\%$), and which show no indication of consecutive spots of comparable brightness aligned along Io's orbit footprint (the structured arc extending westward of Io, if related, is much fainter). Accommodating our new observations with the apparent structural complexity of the radio source will be a major challenge.

In the standard ionospheric interaction model¹, the implication of a low reflection coefficient, $R = (1 - \Sigma_j/\Sigma_A)(1 + \Sigma_j/\Sigma_A)$ would be that the ionospheric conductance Σ_j (hitherto fairly unconstrained) must well match the wave conductance Σ_A (which is well known). Another important consequence is that any microphysical model of Alfvén-wave interaction must now be able to account for the acceleration of such a large electron flux.

Comparing the power input with the power radiated by the most intense radio bursts from the IFT, $\gg 10^9$ W (ref. 17), we infer also that the mechanism converting particle energy into

FIG. 1 Left, images of the FUV Jovian aurorae taken by the HST Faint Object Camera. The bright disk is due to Rayleigh-scattered solar flux, and the dark belt in the south to clouds produced by the impact of comet Shoemaker-Levy 9. The black elongated features are coronagraphic fingers. Jupiter rotated by $\sim 7^\circ$ during exposures. Spatial resolution permits to detect the FUV Io footprint (in the south) (see also ref. 21). A two-dimensional spatial-filtering code improves visibility of the limb and of real features. The images are rebinned into 512×512 arrays of $(0.028 \text{ arcsec})^2$ -pixels, and an oblate spheroid is fitted to the disk outside the aurorae. Right, magnified view of the spot. It extends from $(-62.75^\circ, 111.25^\circ)$ to $(-63.15^\circ, 114.25^\circ)$ (latitude, phase angle from the anti-Earth direction) (between crosses). Uncertainties in the offset between images, limb fitting and spot location are within $\pm 2.25^\circ$ and $\pm 0.6^\circ$ respectively (4–5 pixels). An emission altitude of 200–800 km above the “surface” (800 eV–150 keV incident electrons in the model of ref. 5) increases the phase angle by 0.3–1.1°. Io is at longitudes $\sim 126^\circ$ and $\sim 135^\circ$ for the south and north images, near the centre of the plasma torus. Its phase is $\sim 89.6^\circ$ and $\sim 93.2^\circ$, respectively (near the dawn meridian), and the calculated phases of its magnetic footprints vary from 102.3° to 105.2° , and 79.4° to 79.17° , respectively, during exposures. The south FUV-spot is therefore 9.6°



($-2.45^\circ, +2.75^\circ$) ahead of the model footprint in the direction of Io's motion. The magnetically conjugate north spot is not detected, despite a very dark background and the high expected signal-to-noise ratio. This suggests it is still beyond the limb, $< 10.5^\circ$ from the IFT footprint (considering the emission altitude might decrease this upper limit down to $3-7^\circ$). Both values are significantly smaller than the value derived from the H_2 emission¹³. Additionally, a tenuous westward arc originating near the spot may suggest some very faint interaction extending $\sim 20^\circ$ (~ 40 Io radii) in the direction of Io's motion.

electromagnetic waves must be more efficient even than for the Earth's auroral kilometric radiation ($> 1\%$).

Finally, the energy flux input through the IFT, $\sim 10-15 \text{ W m}^{-2}$, is enormous (equivalent to the total Earth's auroral power concentrated in a $60 \times 200 \text{ km}$ area), and must trigger violent local plasma and atmospheric processes.

Knowledge of the exact location of the spot is also of importance in the determination of a number of magnetospheric parameters which are involved in the Io/inner-magnetosphere interaction. The Io-generated signature should be carried by an Alfvén wave whose propagation speed along the field, $V_A = B/(\mu_0 \rho)^{1/2}$, depends on the field-strength B and the local plasma mass-density ρ . Meanwhile, the magnetic field line rotates

past Io with a relative velocity of 57 km s^{-1} , so that the wave reaches the ionosphere ahead of Io's footprint. The value of the lead angle is thus controlled by the high-density, low-field regions of the Alfvén-wave path through the torus. A Voyager-derived model of the Io torus¹⁸ supports displacements of a few degrees ($\leq 8^\circ$) (refs 1, 8).

We have taken considerable efforts to fit the limb of the planet and estimate all possible uncertainties (Fig. 1 legend). This leads us to locate the centre of the spot at System III (SIII) magnetic longitude 102.85° ($-2.45^\circ, +2.75^\circ$), latitude $63^\circ \pm 0.6^\circ$, slightly poleward of the model footprint of Io's orbit, in close agreement with the locus of the IFT determined by Connerney *et al.*¹³ (Fig. 2).

We follow Connerney *et al.* in comparing the position of the

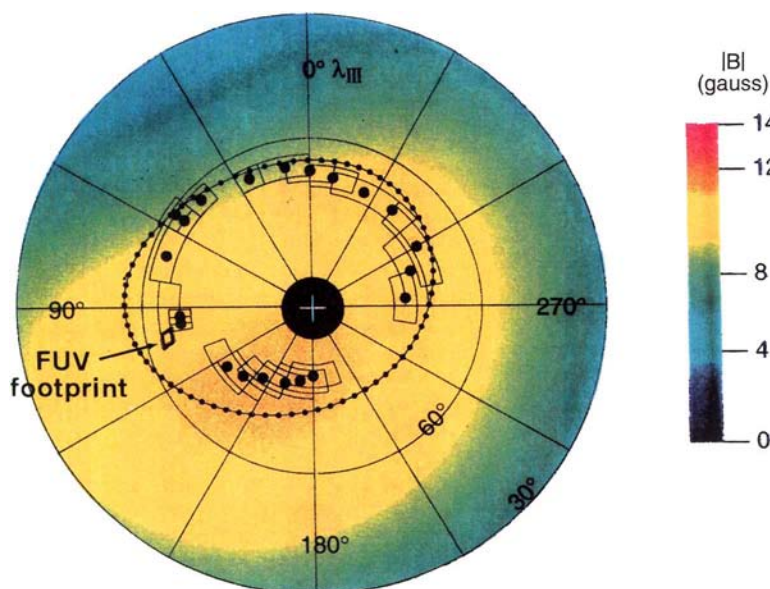
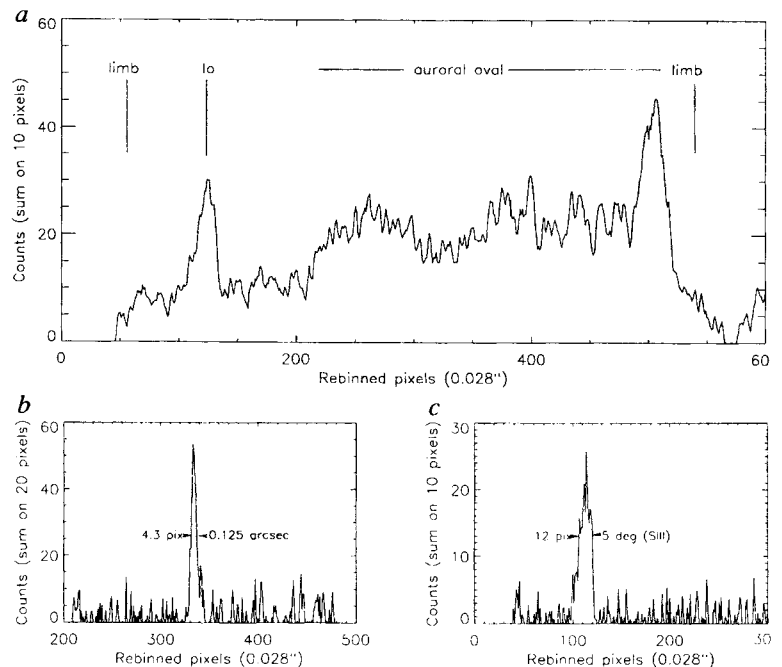


FIG. 2 Polar plot of the footprint of the IFT. The boxes and circles are the infrared spots observed¹³ in 1992 with their error boxes, the parallelogram is our FUV spot (including error bars), and the dotted curve is the theoretical footprint of the orbit of Io in the Q_6 magnetic-field model. We see that the ultraviolet signature we have detected is well within the locus of the infrared signatures. Both are significantly poleward of the model footprint at the longitude of interest, hence there is some uncertainty in the models near the “surface” of Jupiter which may affect the determination of the lead angles in absolute value, but not their relative value at any given location. The infrared spots used in ref. 13 to determine the lead angle are those closest to the ultraviolet spot.

FIG. 3 a, Plot across the track of the Io footprint and the aurora (dashed line in Fig. 1), smoothed over 2 pixels. Abscissa are (rebinned)-pixel numbers, ordinates are counts per pixel along the plot (derived from a sum over the number of pixels indicated perpendicular to the plot). The signature of Io is at about the 12σ level, and 65–120% of the auroral signal. b, c, North–south and east–west plots (respectively) across the track, with the disk background subtracted. Both plots display very sharp gradients. At half-maximum, the spot extends over 12 pixels in the direction of motion, that is $\sim 5^\circ$ in longitude, and 4.3 pixels (0.12 arcsec) in the transverse direction, that is, $\sim 0.9^\circ$ in latitude. This is in fair agreement with the size of the IFT footprint (2×0.5 pixels projected on the image, or $0.5^\circ \times 0.1^\circ$), its motion during the exposure ($\sim 3^\circ$), and the PSF, and is consistent with 75% of the spot energy originating from a region within ~ 5 Io radii from Io's surface (not corrected from noise contribution and any dispersive spreading of the Alfvén wave¹²). The peak brightness corresponds to 0.7×10^6 rayleigh of total emission in the FUV H_2 Lyman–Werner bands²², comparable to auroral brightnesses. By contrast, the infrared spot¹³ was significantly fainter than the nearby auroral emissions, presumably due to spreading of the emission by a larger PSF. Converted into power units, the total flux is 5×10^{40} W radiated in the FUV H_2 emission. A code of energy degradation of energetic charged particles precipitating in the Jovian atmosphere⁵ allows us to compute the fraction of the incident energy lost in excitation of the H_2 bands. This fraction ranges from $\sim 1/4$ to $\sim 1/6$ for 1–200 keV electrons and 25–500 keV protons, leading to a total flux of $(2-3) \times 10^{41}$ W m⁻² carried down to the IFT by the energetic particles flowing along the IFT.



spot with the instantaneous footprint predicted by the O_6 magnetic-model¹⁵. These workers found the spot leading the IFT by $15-20^\circ$, but our lead angle is only $7-12.3^\circ$. The apparent consistency with model predictions cannot be trusted because the magnetic field is still uncertain by a few degrees. By contrast, the difference between the two measurements is of importance. Both datasets were obtained with Io at almost the same longitude ($\sim 120^\circ$), so that neither uncertainties in the field model nor Io's position in the torus can explain this discrepancy. We now consider other possibilities. (1) Uncertainties in the location of the FUV spot have been carefully estimated. Additionally, a small lead angle is also derived in the north, where we fix an upper limit of $7.5^\circ \pm 3^\circ$ (Fig. 1). By contrast, although the infrared spot is less well defined, much fainter than and not well resolved from nearby aurora, several independent observations were used. We doubt that the results can be reconciled within experimental uncertainties. (2) The generation mechanisms of the infrared emissions must in principle be distinguished from those responsible for the FUV. H_3^+ emissions are believed to be mainly thermalized, due either to collisional heating of the atmosphere by particle precipitation or to Joule heating, rather than being a direct signature of collisional excitation, like the FUV emission. The ultraviolet and infrared signatures are unlikely to separate, however, as here the precipitating particles carry a large portion of the current responsible for Joule heating, and because the absence of strong H_3^+ hot-bands shows that thermalization is rapid. (3) Temporal effects therefore remain the only source of variability. A second set of images obtained on 13 July, with Io at nearly the same longitude, and with the IFT model footprints also just beyond the limb, shows no sign of any bright spot. This sets an upper limit of the lead angle at that date of $7^\circ \pm 3^\circ$ in the south and only $4^\circ \pm 3^\circ$ in the north, even less than on 9 August.

Up to now, the separation between the magnetic footprint of Io and its "auroral" footprint had been assumed to have a single particular value (still to be determined) at any given location, and any discrepancy between datasets was attenuated to observational uncertainties. The above discussion now strongly suggests that this separation is intrinsically variable. Local increases or decreases by a factor of four of the mass density in the plasma torus may lead to the reported variations of ~ 2 . Such density variations could either result from local corotating density variations (like those discov-

ered recently by Ulysses from the narrow-band kilometre-wave radio emission), or from time-varying volcanic activity, ionization of Io's atmosphere, or composition in the vicinity of Io (molecular ions).

Precise understanding of the mechanisms at work in the Io–Jupiter interaction will come only from long-term monitoring of the brightness and lead-angle variabilities, in particular of their dependence on Io's SIII longitude, of their north–south asymmetry, and of their temporal variability. This study thus opens up the possibility, in the longer term, of monitoring the source and transport of plasma in the inner magnetosphere (more accurate magnetic field models will soon become available), and the ionospheric conductivity, two important inputs for magnetospheric models. □

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Cationic cyclopropanation by antibody catalysis

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REACTIONS involving highly reactive carbocations play a central role in many important chemical processes, such as cyclization reactions^{1,2}. However, the potential for controlling the pathways of such reactions to obtain energetically disfavoured (but desirable) products has been hard to realize because of the difficulties inherent in controlling the conformation and chemical environment of the carbocation intermediates. Antibody catalysts, with their high specificity and binding energies, can provide the degree of conformational and chemical control necessary for directing such reactions^{3,4}. Here we show how antibody catalysis can guide cationic cyclization reactions selectively to form products (in high yield) that would otherwise be highly disfavoured. Most notable is the formation of a strained bicyclic compound containing a rare cyclopropane group. To explain our results, we propose a common reaction scheme in which the key step is the formation of a highly reactive protonated cyclopropane intermediate; subtle structural modifications to the substrate (the compound on which the catalytic antibody acts) lead to dramatic differences in the structure of the final product.

Apart from their use in synthesis, cyclopropanes have played an important role in the understanding of reaction routes involving carbocations through the participation of three-centre, two-electron bonds⁵⁻⁷. Experimental verification of the existence of protonated cyclopropanes was central to discussions concerning classical versus non-classical carbocations^{8,9}. Even though they have played a central role in the theory and practice of organic chemistry, there is no general method to enforce the stereoelectronic parameters that favour cyclopropane formation in cationic reactions. Indeed, although they are often formed in biotic reactions involving carbocations¹⁰⁻¹², in over forty years of study they have only rarely been seen in abiotic cationic processes¹³⁻¹⁶.

Antibodies are produced by living cells in response to the presence of a foreign species, and can be isolated and raised in large quantities. For antibody catalysis, the key is to raise an antibody that will bind to a specific reactant molecule in such a way that the resulting conformational and electronic changes direct

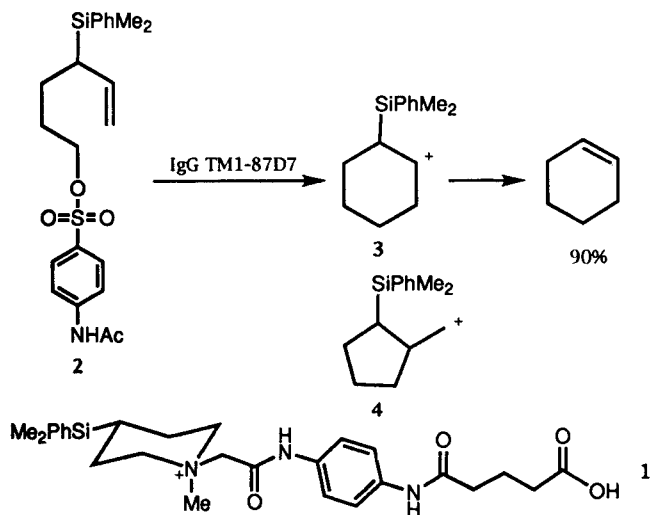


FIG. 1 Cationic cyclization reaction catalysed by antibody TM1-87D7.

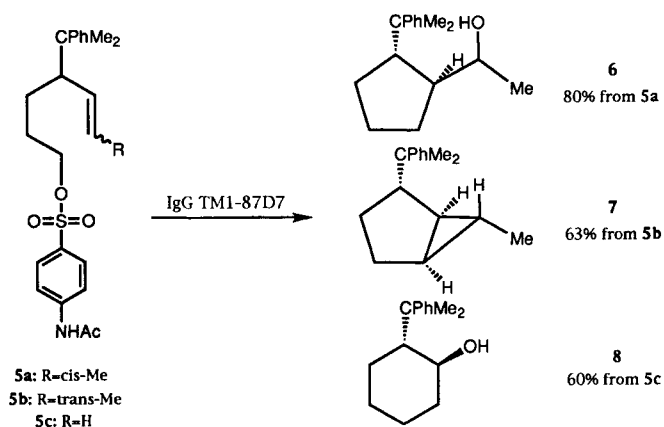


FIG. 2 Reaction scheme displaying substrate specificity and the ability of a single antibody to control the reaction route of three different olefins.

the outcome of the reaction. We previously demonstrated that the antibody TM1-87D7, raised against the hapten 1 (a quaternary ammonium salt) will catalyse a cationic cyclization reaction with substrate 2 to produce cyclohexene^{17,18} (Fig. 1). This reaction has a highly reactive carbocation intermediate, whose fate is largely controlled by the antibody catalyst. But substrate 2 also has intrinsic chemical features that play a role in guiding the reaction to produce the more stable secondary carbocation 3 rather than the primary carbocation 4. We therefore envisaged that by making subtle structural modifications to the substrate, we could intervene in the reaction to direct the formation of specific products. Thus the silicon atom of the phenyldimethylsilyl group could be replaced with a carbon atom so that a potential β -effect would no longer influence the reaction pathway¹⁹. Also, a methyl functionality could be added to the terminal olefin appendage of 2, thereby eliminating the chemical advantage of a reaction route involving a secondary rather than a primary carbocation¹.

Based on the above assumptions, the *cis*-olefinic substrate 5a was synthesized (Fig. 2). When 5a was incubated with IgG TM1-87D7, clean formation of a single diastereomeric exocyclic alcohol 6 was observed (Fig. 2; the catalytic constant, $k_{cat} = 0.013 \text{ min}^{-1}$, and the Michaelis constant, $K_m = 58 \mu\text{M}$, 85% hexane/15% bis-Tris, pH 7.0, 25 °C). All product yields are based on consumed starting materials, and no product inhibition was observed in any of the cases described. The structure of 6 was confirmed by comparing both the proton NMR spectrum and the gas chromatography trace of the sample obtained from the antibody-catalysed reaction with a racemic sample synthesized independently (synthesis not shown). But the most remarkable result was obtained when the *trans*-olefin 5b was used as a substrate: formation of 7 in 63% yield was observed (Fig. 2, $k_{cat} = 0.021 \text{ min}^{-1}$, $K_m = 102 \mu\text{M}$). Importantly, the rate accelerations achieved by this antibody catalyst are within an order of magnitude of those of natural enzymes that catalyse similar cationic processes²⁰. The most salient feature of 7 is the strained bicyclic ring found in the bicyclo [3.1.0] hexane unit. As with 6, we confirmed the structure of 7 by direct comparison of both the proton NMR spectrum and the gas chromatography trace of the sample obtained from the antibody-catalysed reaction with a synthetic sample obtained according to the scheme shown in Fig. 3. To confirm that the cyclopropanation reaction was dependent on antibody catalysis, substrate 5b alone was subjected to Johnson-like conditions (formic acid/sodium formate, 80 °C, 2 h; ref. 2) or conditions somewhat comparable to those used for antibody catalysis (hexane/bis-Tris, pH 2, 80 °C, 36 h, or hexane/dimethylsulphoxide/bis-Tris, pH 2, 80 °C, 12 h). In the absence of antibody, the reaction conditions were necessarily much more drastic, because otherwise the uncatalysed reactions could not be observed. Even

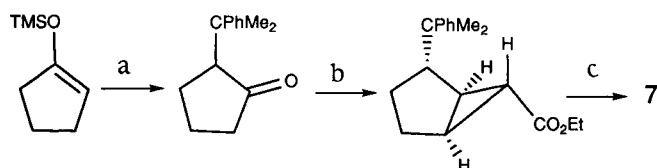


FIG. 3 Synthesis of the bicyclo [3,1,0] hexane **7**. Conditions: a, 2-phenyl-2-propylacetate, ZnCl_2 , yield 93%; b, (three-step reaction sequence) (1) *p*-toluenesulphonylhydrazide, (2) *n*-BuLi, (3) ethyl diazoacetate, CuSO_4 , 30%; c, (three-step reaction sequence) (1) lithium aluminium hydride, (2) methansulphonyl chloride, (3) lithium triethylborohydride, 76%.

under these harsh conditions, a cyclopropane product could not be detected, demonstrating that antibody catalysis is essential for its formation.

To investigate the importance of olefinic substitution in the antibody-catalysed reaction, we also studied the cyclization of compound **5c**. Here only the silicon atom was replaced (compare with substrate **2**) and, as anticipated, cyclohexanol **8** was the major product (Fig. 2; $k_{\text{cat}} = 0.01 \text{ min}^{-1}$, $K_m = 31 \mu\text{M}$). Although broad substrate utilization was observed, its fidelity is still preserved in that all reactions were competitively inhibited by the hapten **1**, showing that the catalysis is taking place in the antibody combining site (for compound **5a**, $K_i = 1.6 \mu\text{M}$; **5b**, $2.4 \mu\text{M}$; **5c**, $1.0 \mu\text{M}$). It is important to note that no cyclized products were observed in the uncatalysed reactions of **5a–c**, again demonstrating the unique ability of the antibody catalyst to control the reaction routes of this cationic process.

A unified reaction pathway invoking protonated cyclopropane **9** (Fig. 4) can be formulated to rationalize all the reaction products observed with different substrates for the antibody-catalysed reaction (Fig. 2). Thus for substrate **5c**, addition of water to **9** at the α -carbon leads to the formation of cyclohexanol **8**. In the case of substrates **5a/b** (which contain an electron-donating methyl group), products **6** or **7** are formed respectively by addition of water to the β -carbon of **9**, or loss of a proton from **9**. The observed product distribution (Fig. 4) can thus be ascribed to control of the central carbocation intermediate by the antibody catalyst. In essence, we see that subtle structural changes in the substrate can yield control of the chemical transformation to the antibody molecule such that alternative reaction routes can be traversed.

The antibody-catalysed cationic cyclopropanation reaction is important from two points of view. First, the cationic cyclopro-

panation reaction is usually a highly disfavoured process in chemical systems. To the best of our knowledge, cyclopropane formation has not been reported in the numerous attempts to mimic 2,3-oxidosqualene cyclase using chemical systems²¹. Indeed, our failure to see the cyclopropane product in solvolysis experiments with **5b** without catalytic antibodies again illustrates the difficulty in obtaining this product.

Although the ability of the antibody to catalyse cyclopropane formation is an important feat from the purely chemical point of view, it can also serve as a model for the study of naturally occurring enzymes that catalyse similar reaction pathways. For example, the first step in the squalene biosynthesis cascade involves the formation of a cyclopropane by the formal addition of a carbon cation to a π -bond²². The mechanistic details of this interesting enzymatic process remain unclear, partly because of the difficulty encountered in the handling of this membrane-bound protein²³. The ready availability of our catalytic monoclonal antibody and the simplicity of our system could provide some insight as to how a protein catalyses cyclopropane formation.

Our work may also provide greater insight into the cationic cyclization/rearrangement process by which cholesterol is synthesized from acetate. Although this is one of the most important pathways in biology, a detailed understanding of the mechanism of this cationic cyclization reaction has remained elusive²⁴. One reason is that polyene cyclization is so complex that it is difficult to determine how a natural catalyst contributes to the requisite initiation, propagation and termination steps of this process. In contrast, antibodies as customized catalysts can be designed to give specific information about the individual parts of an otherwise complicated cascade. Thus the catalyst we describe here can be considered a specific initiator and terminator that is capable of starting and ending a polyene cyclization reaction in a highly specific manner. Complexity may be added to catalysts in the future so that the antibody molecule might be used to control the energy of the various carbocations and high-energy intermediates that appear along the reaction coordinate, thereby directing the chemical outcome of more complex polyene cyclization reactions. In particular, because catalytic antibodies can facilitate disfavoured chemical events³, we should be able to reroute cyclization reactions by overcoming the unfavourable barriers intrinsic to anti-Baldwin³ and anti-Markovnikov²⁵ processes (These are chemical rules governing ring-closure reactions and the regioselective addition to unsaturated substrates, respectively). This should lead to unusual steroids which could have important applications in organic synthesis and medicine. □

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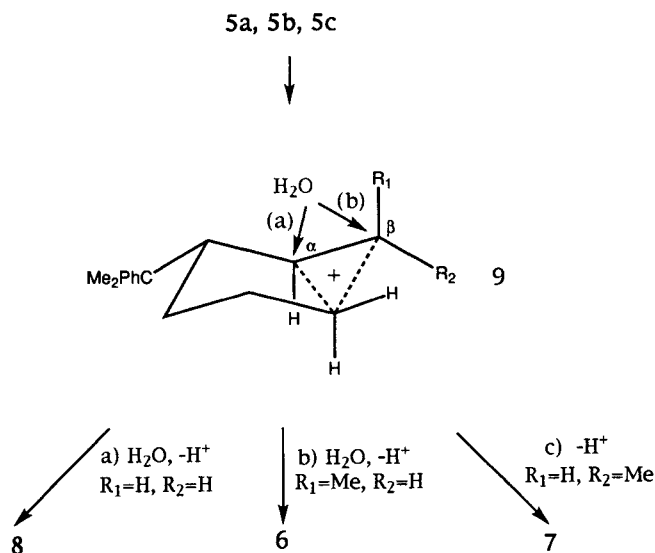


FIG. 4 A unified reaction scheme that formulates the different reaction outcomes shown in Fig. 2.

Recent atmospheric warming and retreat of ice shelves on the Antarctic Peninsula

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IN 1978 Mercer¹ discussed the probable effects of climate warming on the Antarctic Ice Sheet, predicting that one sign of a warming trend in this region would be the retreat of ice shelves on the Antarctic Peninsula. Analyses of 50-year meteorological records have since revealed atmospheric warming on the Antarctic Peninsula^{2,3}, and a number of ice shelves have retreated⁴⁻⁸. Here we present time-series of observations of the areal extent of nine ice shelves on the Antarctic Peninsula, showing that five northerly ones have retreated dramatically in the past fifty years, while those further south show no clear trend. Comparison with air-temperature data shows that the pattern and magnitude of ice-shelf retreat is consistent with the existence of an abrupt thermal limit on ice-shelf viability, the isotherm associated with this limit having been driven south by the atmospheric warming. Ice shelves therefore appear to be sensitive indicators of climate change.

Ice shelves fringe most of the Antarctic continent where there are bays or islands to constrain them. Robin and Adie⁹, however, noted that a portion of the Antarctic Peninsula was free of ice shelves, despite many glacier-fed bays and offshore islands. The limit of ice shelves apparently corresponded with the 0 °C January isotherm, and they concluded this marked a "limit of viability". Other constraints on ice-shelf viability based on ocean temperature¹⁰ and tidal amplitude¹¹ have been proposed, but have not been shown to fit the known distribution.

Where there is little summer melting, the yearly surface temperature cycle is attenuated to ~5% at a depth below the ice surface of 10 m (ref. 12). The 10-m temperature thus provides an estimate of the mean annual air temperature. Mercer¹ suggested that the downward percolation and subsequent refreezing of surface melt could eliminate the cold thermal wave from the previous winter and raise the ice shelf to the pressure melting point throughout. The so-called temperate ice shelf thus created was at that time thought to be inviable. However, it is unlikely that this process would be sufficient to form a temperate ice shelf if percolation is restricted to the near surface layers (~10 m). Furthermore, there is strong evidence that portions of stable ice shelves can approach the temperate state¹³. Nevertheless, we believe that increased melt could play an important role in providing the reason for a climate-imposed limit.

The duration and extent of the summer melt season has been determined from satellite passive microwave data^{14,15}. The threshold for melting in the Antarctic Peninsula region is about -2.5 °C (monthly average) and the area of melt increases rapidly with temperature¹⁴. Trends are not well established, being masked by the inter-annual variability from 1978 to 1987¹⁴, but the number of days per year with summer melting seems to have increased (one day per year) over the period 1978-91 on four ice shelves on the Antarctic Peninsula¹⁵.

The only climate parameter that is well mapped on the Antarctic Peninsula is the mean annual air temperature¹⁶ (Fig. 1). The distribution of ice shelves indicates that the -5 °C mean annual isotherm in Reynolds' compilation¹⁶ could be taken as proxy for the limit of viability of ice shelves. Although the 0 °C January isotherm is not well mapped, the sparse data available suggest that it coincides with the mean annual -5 °C isotherm¹⁷. For example, mean January temperatures at Faraday Station are around 0.5 °C, whereas the mean annual temperature is -4.4 °C

(ref. 2). We thus adopt mean annual air temperature as the best available indicator of the amount of summer melt.

Meteorological records along the west coast of the Antarctic Peninsula^{2,3} show a spatially consistent warming trend. At Faraday Station, a warming of 0.056 °C yr⁻¹ has been measured² since 1945, a total of ~2.5 °C. Consistency along the west coast is to be expected, as sea-ice extent in the Bellingshausen Sea strongly modulates the temperature of the entire area¹⁸. In 1988-91 sea-ice extent in the Bellingshausen Sea reached a minimum, coinciding with the warmest recorded temperatures on the west coast of the Antarctic Peninsula¹⁹. In contrast, the climate of the east coast is governed by conditions in the Weddell Sea¹⁷ and there is little meteorological evidence that warming has also occurred on the east coast. The temperature record from Marambio Station is short and incomplete²⁰, and Jones' comparison of a two-year record from Snow Hill Island with spatially smoothed mean temperatures 1957-75 (ref. 21) is untrustworthy, because the area has extremely high spatial gradients of mean annual air temperature.

Figure 2 presents a catalogue of changes in ice-shelf areas on the Antarctic Peninsula since direct observations began, compiled from published sources^{4-8,22-27} and recent satellite imagery. How do we distinguish climate-induced retreat from normal calving? We suggest that an ice shelf which is no longer viable will suffer a progressive retreat, via a series of small calving events occurring each year over a period of many years, without substantial readvance. Such behaviour was clearly seen during the disintegration of Wordie Ice Shelf (Fig. 3), and Fig. 2 suggests similar behaviour in three other ice shelves close to the -5 °C isotherm; namely, the ice shelf that occupied Prince Gustav Channel, Larsen Inlet, and the northernmost section of Larsen Ice Shelf (Sobral Peninsula to Robertson Island, hereafter Larsen-A). An order of magnitude smaller and potentially quite different, Müller Ice Shelf has shown a progressive retreat since the 1950s, but without complete disintegration. In summary, each retreat was pro-

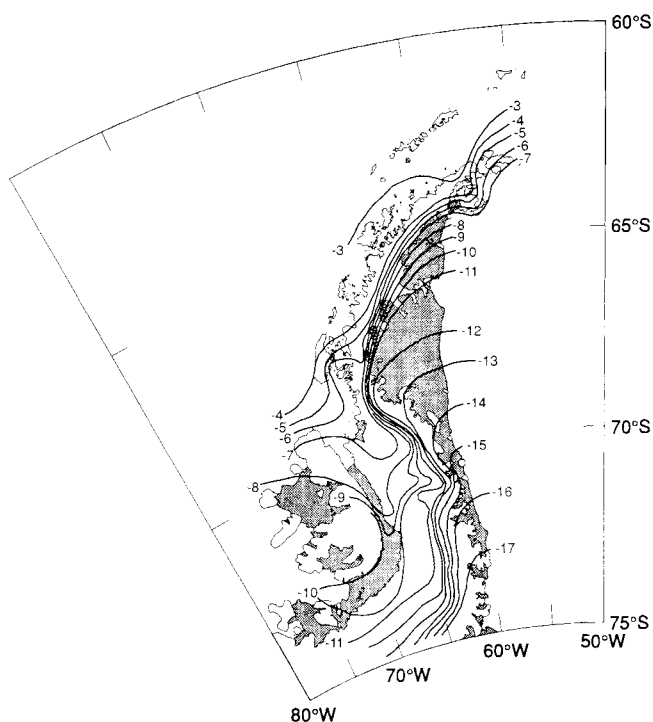


FIG. 1 Map of pre-1981 mean annual air temperatures in °C. Derived from temperatures 10 m below the ice surface, normalized to sea-level by Reynolds¹⁶. Ice shelves (shaded) are indicated at their mid-1970s extent.

gressive, continuing over several decades without substantial readvance, and all these ice shelves are now at their minimum recorded extent. Even if calving ceased entirely, many decades of readvance would be required to restore any one ice shelf to its former extent.

Ice-shelf retreat has occurred on both coasts of the Antarctic Peninsula; this is strong evidence that the warming trend is, indeed, present on the east coast. Those farther south of the -5°C isotherm have shown little change in extent (for example, Wilkins Ice Shelf, Bach Ice Shelf and "Larsen-C"—Jason Peninsula to Gipps Ice Rise).

The surviving section of Larsen Ice Shelf, "Larsen-B" (Robertson Island to Jason Peninsula), is now closest to the apparent limit. Thus far it has not shown the diagnostic progressive retreat, but did suffer a major calving event in early 1995 and airborne reconnaissance has revealed rifts opening behind the new ice front (M.R.A. Thomson, personal communication) possibly pre-saging imminent progressive retreat.

Comparison of mean annual air temperature with the magnitude of warming trend observed by King² (2.5°C since 1945) shows that the pattern of retreat is consistent with a southerly migration of a limit of viability (the -5°C isotherm) driven by the atmospheric warming. Reynolds' contouring¹⁶ of the mean annual

air temperature took no account of temporal trends in temperature, but because most of the data were collected in the 1970s, removing the temporal trend would not significantly alter the distribution, and we take it as a reasonable estimate of the isotherms for the mid-1970s. Furthermore, we assume that all areas have warmed by the same amount and so the application of a simple bias to Reynolds' map will give the temperature distribution over the past 50 years.

It appears that during the 1940s and 1950s the limit of viability was in the position marked by Reynolds' -5°C isotherm. A 2.5°C warming since that time would have shifted the limit roughly to the position of Reynolds' -7.5°C isotherm (Fig. 1). On the west coast this corresponds to a migration from near Müller and Jones ice shelves, to south of Wordie Ice Shelf, all of which have retreated. Further warming of 1°C might threaten George VI Ice Shelf and Wilkins Ice Shelf. On the east coast gradients of mean annual air temperature are steeper, and a similar warming would have caused a southerly migration of perhaps only 50 km. Reynolds' -5°C isotherm lies north of Prince Gustav Channel, and the -7.5°C isotherm passes south of Sobral Peninsula; the migration of the -5°C isotherm crossed two ice shelves which have since disintegrated. The recent retreats farther south imply either inadequacy of the isotherm map or a stronger warming trend.

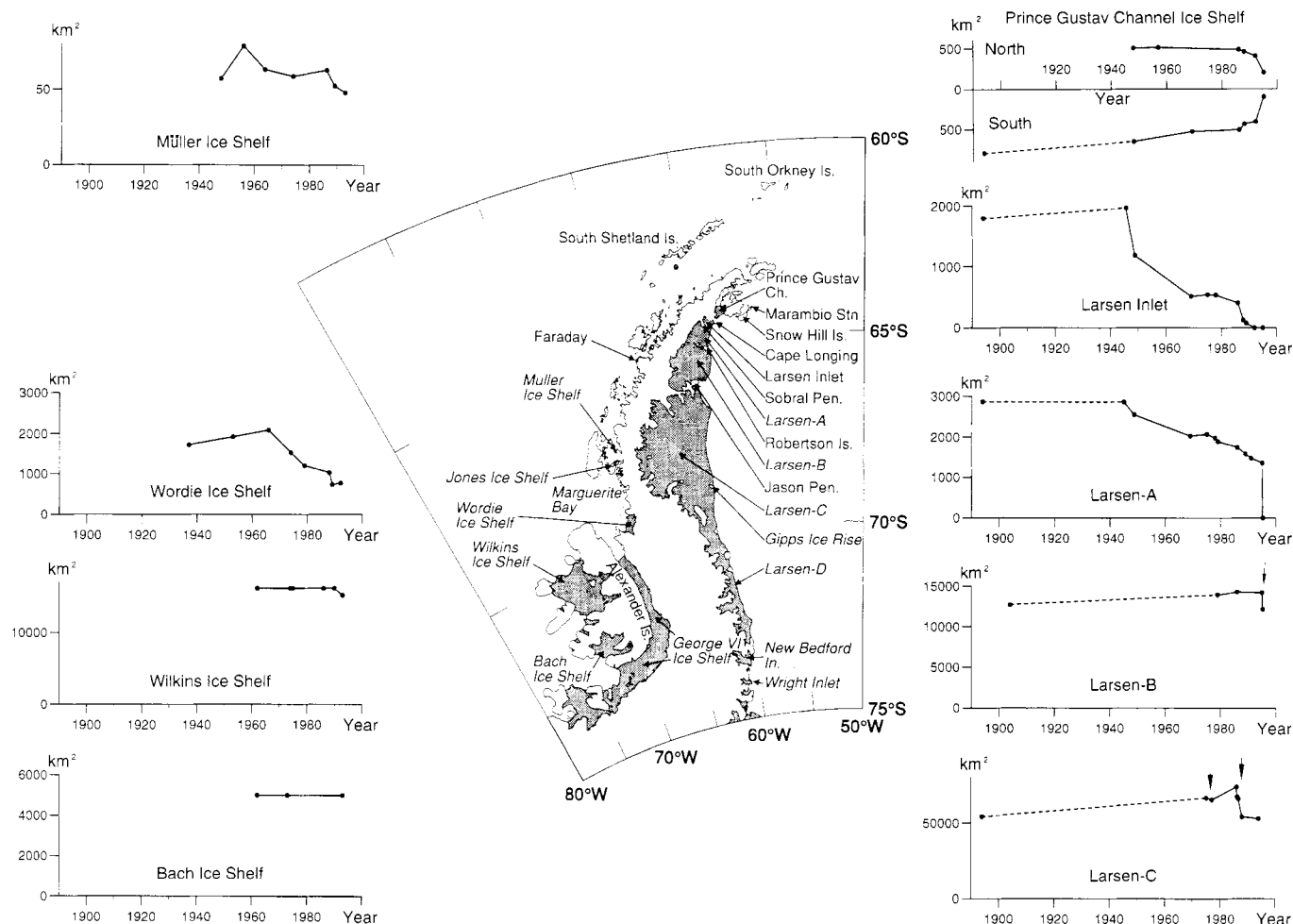


FIG. 2 Catalogue of changes in ice-shelf extent (in km^2) on the Antarctic Peninsula. The total area of each ice shelf was calculated using various sources of ice-front position but using the same grounding line. Dotted lines indicate that significant fluctuations were probable in these intervals. Prince Gustav Channel has two ice fronts and was divided arbitrarily into northern and southern sections. Documented large calving events on Larsen Ice Shelf are marked by arrows. The tiny Jones Ice Shelf is known to have retreated since the 1950s (A. Fox, personal communication) but the data

are inadequate to allow detailed study. Larsen-D, New Bedford Inlet and Wright Inlet have shown little change in area in the past two decades. The trend of the northern front of George VI Ice Shelf is unclear, showing ~ 10 km of retreat between 1949 and 1974²² but then remaining largely stable. Warming has been noted in meteorological records from South Shetland Islands, Faraday Station and Marguerite Bay², and from Alexander Island³.

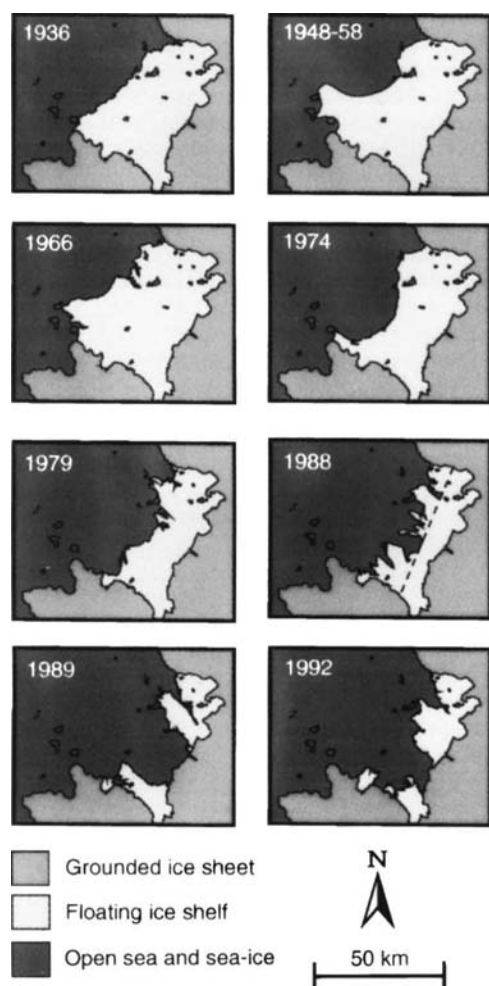


FIG. 3 Cartoon of 50 years of retreat of Wordie Ice Shelf ($69^{\circ}11'S$, $67^{\circ}30'W$) from 1936 to 1992. The area shown is approximately $90\text{ km} \times 70\text{ km}$. Features marked within the ice shelf are areas of grounded ice. The ice-shelf front advanced a little between 1936 and 1966, but then retreated from 1966 to 1974. Between 1979 and 1988 many longitudinal rifts appeared in the ice-shelf surface⁴. From 1989 to the present, the ice shelf is little more than three disconnected ice tongues. Dashed line in 1988 panel indicates the limit of available ice front data.

A common feature of the ice-shelf retreats is marked acceleration during the final stages. Most dramatic was the final phase of collapse of Larsen-A, a loss of $1,300\text{ km}^2$ in 50 days in which the ice shelf broke up into thousands of small icebergs producing a plume 200 km into the Weddell Sea⁸.

We are still uncertain what mechanisms are important during ice-shelf retreat, although several can now be postulated. Mercer's original notion, the removal of the winter cold wave by refreezing of melt water, allows a sub-surface magnification of a small air temperature rise. However, simple conduction could transmit such a temperature change through the ice shelf only slowly; a rise in temperature of 5°C at 10 m depth would cause around 1.2°C warming at 60 m depth after 50 years. The temperature-strength (fracture toughness) relation for ice is uncertain and it is unclear whether this is sufficient to cause the ice shelf to weaken enough to retreat. Alternatively, if surface melt water were to percolate deep into the ice shelf, either through inter-crystal veins or through crevasses and moulins, it might reduce the strength of the ice directly²⁸. It is also possible that a small warming at the surface changed surface mass balance from accumulation to ablation, altering its thermal structure, and preventing lines of

weakness, such as crevasses, being healed by yearly accumulation. This is a distinct possibility for Wordie Ice Shelf which showed a clear increase in surface crevassing between 1974 and 1979.

Climatic forcing may not need to be very strong to produce ice-shelf retreat. Between periods of retreat, ice fronts generally hang on ice rises/rumples or at the mouth of a bay. Decoupling of the ice front from grounded points removes their restraint and allows the ice shelf to break back rapidly to the next stable configuration, possibly triggered by ocean swell²⁹; Wordie Ice Shelf certainly retreated in stages between sets of pinning points (Fig. 3). Thus even a small retreat driven by increasing temperature may have led to the rapid collapse we have seen during the final phase of retreat.

Given continued warming, Larsen-B will probably continue to retreat, and eventually Larsen-C may well behave in a similar way. Prediction is, however, less certain on the west coast. Wilkins Ice Shelf is sustained mostly by *in situ* accumulation³⁰, so its initial response to climate warming may be a thickening as accumulation increases. George VI Ice Shelf has a very small ratio of calving front to ice-shelf area and loses most of its mass by basal melting³¹, and may be relatively insensitive to changes in surface temperature.

On millennial timescales these retreats may not be unique or even unusual; ice-shelf fronts may have oscillated several times during the Holocene¹⁰. But in the short term Mercer's predictions have been borne out, and the spatial and temporal pattern of ice-shelf retreat is similar to that he proposed. We conclude that ice-shelf extent may well be a sensitive indicator of regional climate change. The pattern of retreat provides evidence of warming in both climate regimes on the Antarctic Peninsula, but due to the high spatial gradients of mean annual air temperature, the warming was achieved by a modest migration of the climate pattern. We have still, however, to determine the precise mechanisms whereby the atmospheric warming had such a catastrophic effect on the ice shelves of the Antarctic Peninsula but it is clear that ice shelves cannot survive periods of warming that last more than a few decades.

We cannot determine whether the Antarctic Peninsula warming can be ascribed to a global warming magnified by regional temperature/sea-ice feedback, or if this is a natural oscillation as a result of the same feedback. We offer no prediction that the warming will continue, but if it does, other ice shelves are threatened. The Filchner-Ronne and Ross ice shelves, which may stabilize the West Antarctic Ice Sheet³², are not immediately threatened by this mechanism, as it would require a further warming of 10°C before the -5°C mean annual isotherm reached their ice fronts.

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Glacial isostatic adjustment and the anomalous tide gauge record of eastern North America

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SEA-LEVEL variations, as recorded by the global network of tide gauges, represent a rich data set for studying a wide range of natural and anthropogenic phenomena, such as the sea-level rise induced by possible global warming. For this purpose, long-term sea-level trends must be corrected for the 'contaminating' effects of continuing glacial isostatic adjustment^{1–5} (GIA). The numerical correction procedure has, for sites on the east coast of North America, yielded a set of highly anomalous sea-level rates characterized by systematic geographical trends^{2,4,5}. We demonstrate that these trends are a consequence of inadequacies in the previous 'standard' numerical prediction for GIA. In particular, we find that the well-known trends in the GIA-corrected tide gauge rates are eliminated if the lower-mantle viscosity of the Earth model used in the GIA prediction is increased. This result obviates the need to explain the anomalous trend as a manifestation of Gulf Stream ocean circulation⁴ or neotectonic processes².

We analyse the tide-gauge data from the east coast of North America using an iterative least-squares inversion in which the rates of sea-level change obtained from the raw data are taken as the observables, and parameters are estimated representing adjustments to the lithospheric thickness, the viscosity of the upper mantle, and the viscosity of the lower mantle. A common sea-level rate is also estimated. The *a priori* values for the Earth-model parameters were taken from the 'standard' model commonly used to generate GIA corrections^{1,3–5}. This standard Earth model⁶ is characterized by an elastic lithosphere of thickness 120 km, an upper-mantle viscosity of 10^{21} Pa s, and a lower-mantle viscosity of 2×10^{21} Pa s. The elastic structure of the Earth model was given by the seismically determined Preliminary Reference Earth Model⁷. As in previous analyses^{1,3–5}, we adopted the ICE-3G model⁸ for the final Late Pleistocene deglaciation event. Partial derivatives with respect to the Earth-model parameters were determined numerically using sea-level rates calculated⁹ for different parameter values. Our initial solutions clearly indicated that the sea-level rates favoured an increase of a factor of ~ 2.5 in the lower-mantle viscosity relative to the standard-model value. Furthermore, the formal uncertainty in this parameter was fairly small, indicating the high sensitivity of the fit to its value. Adjustments to the other two Earth-model parameters were small, and therefore had relatively little effect on the misfit. These parameters were therefore held fixed to the standard-model values for the inversions reported here. The 'raw' tide-gauge rates, together with the rates corrected using the standard and revised Earth models, are shown in Fig. 1. The scatter in the sea-level rates corrected using the revised model is a

factor of ~ 3 smaller than that for the rates corrected using the standard model (Fig. 2).

The present-day rate of sea-level change due to GIA predicted by the standard model is characterized by a rapid sea-level fall to the northwest, associated roughly with the location of the ancient Laurentide ice sheet, and a moderate ($0\text{--}3\text{ mm yr}^{-1}$) sea-level rise in the 'peripheral bulge' area to the south and east (Fig. 3a). The sea-level fall in Canada is a manifestation of the uplift of the depressed solid surface in that region, whereas the sea-level rise is associated with the subsidence of the peripheral bulge that surrounds the central depression. The standard model predicts that the east coast of North America lies almost exclusively within the peripheral bulge region. Moving north from Florida along this coast the predicted sea-level rise increases monotonically up to a latitude of about 38°N . Between latitudes 38° and 43°N the variation in the predicted sea-level change is more gradual, as the 1.8 mm yr^{-1} contour roughly follows the coast. The curve representing the standard model (Fig. 1a) therefore has a nearly constant value between these latitudes.

We have found that the predicted location of the peripheral bulge is sensitive to changes in the lower-mantle viscosity, whereas variations of the upper-mantle viscosity and lithospheric thickness mainly affect the predicted amplitude of the peripheral bulge on a path traced along the North American east coast, at latitudes

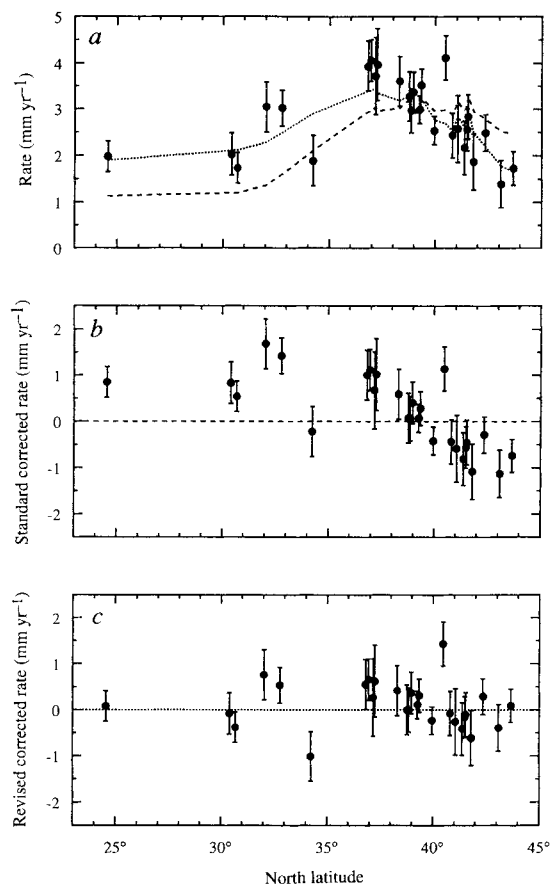


FIG. 1 Rates of sea-level change for the North American sites used in the analysis (error bars, 3σ). a, Raw tide-gauge rates (data points), and the GIA correction (dotted line) which has been shifted for the best-fitting coherent rate ($1.5 \pm 0.3\text{ mm yr}^{-1}$) computed for the revised model. This model is characterized by a lower-mantle viscosity of 4.7×10^{21} Pa s. The analogous correction based on the standard model (defined in text) is shown by the dashed line. b, Residual tide-gauge rates corrected using the standard model, after fitting for a constant rate. c, Residual tide-gauge rates corrected using the revised model and the constant rate term. The residuals shown in parts b and c are related to the values in Table 1 by subtraction of the mean (common) sea-level rate of 1.5 mm yr^{-1} .

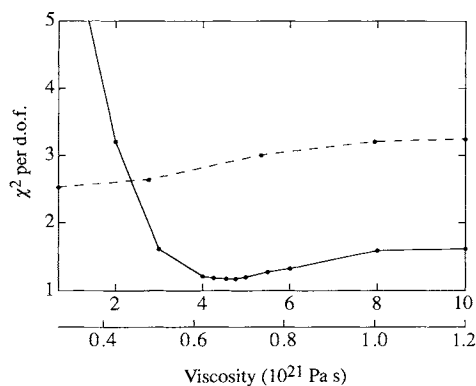


FIG. 2 χ^2 per degree of freedom (d.o.f.) as a function of lower-mantle viscosity (solid line, upper scale) and upper-mantle viscosity (dashed line, lower scale). The minimum χ^2 is achieved for a lower-mantle viscosity of 4.7×10^{21} Pa s. An F -test yields a level of significance of $>99\%$ for the decrease of the minimum χ^2 relative to that for the standard model. We also performed least-square solutions, with no further iterations after the initial adjustment, in which the lower-mantle viscosity and lithospheric thickness were estimated for a range of initial lithospheric thicknesses between 120 and 350 km. The estimated lower-mantle viscosity values were consistent (at the 1σ level) with 4.7×10^{21} Pa s, and the estimated lithospheric thickness values were consistent with, although slightly lower than, the standard value of 120 km.

below 45° N. The sensitivity of the peripheral-bulge dynamics to variations in mantle viscosity has previously been considered in some detail^{10–13}. As the deep-mantle viscosity is increased, the boundary between the central uplift region and the bulge migrates away from the previously glaciated region (Fig. 3a,b), and the predicted sea-level rise due to GIA in the northeastern United States drops significantly. This sensitivity permits the revised Earth model to reconcile the trend evident in the sea-level rates determined from the raw tide gauge record (Fig. 1a,c); the revised model rates, as well as the observed rates, exhibit a decrease significantly sharper than that of the standard model north of 37° – 38° .

It has previously been noted¹⁴ that the standard GIA correction does not reconcile the observed sea-level rate for the Key West site. In fact, the standard model (Fig. 1b) underestimates all except one of the sea-level rates south of 38° . The revised model fits the Key West rate and performs quite well for the southern sites generally (Fig. 1c).

Our simultaneous (revised) estimate of 1.5 ± 0.3 mm yr⁻¹ for the common sea-level rise is significantly different from the

TABLE 1 Mean corrected sea-level rates

North Latitude	Standard corrected rate (mm yr ⁻¹)	Revised corrected rate (mm yr ⁻¹)	Rate uncertainty* (mm yr ⁻¹)
North American east-coast sites			
23°–35°	2.28	1.45	0.16
35°–40°	1.74	1.71	0.13
40°–45°	1.01	1.56	0.15
Far-field global sites			
8°–58°	1.6	1.4	0.4†

* Calculated using the 3σ errors of Fig. 1.

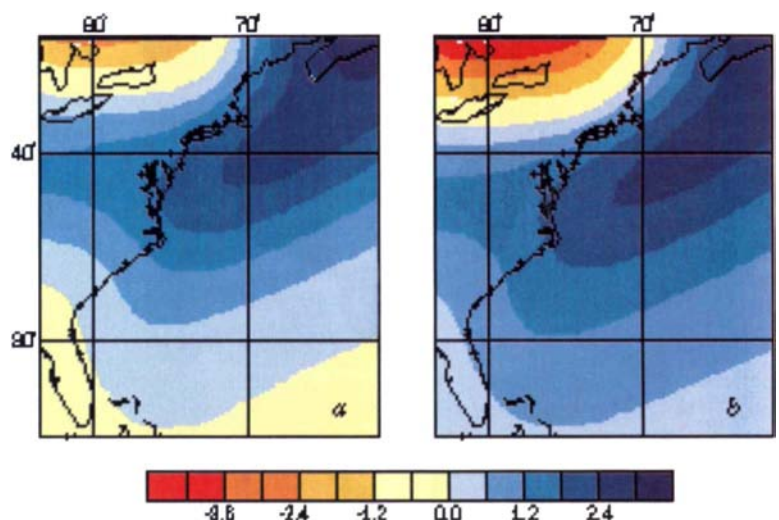
† Based on the weighted r.m.s. variation of the revised corrected rates about the weighted mean.

2.5 mm yr⁻¹ cited by Douglas⁴ for southern sites (south of New York) alone. In that analysis, the northern sites were found to yield a sea-level rise of 1.3 mm yr⁻¹, for a combined east-coast rate of 1.9 mm yr⁻¹. The separation of the data set into southern and northern parts was forced by the significant north–south geographical trend associated with sea-level rates corrected using the standard GIA model⁴ (Fig. 1b). This separation is inconsistent with the notion that the residual rates reflect a globally coherent signal. In this regard, our revised model is consistent with both the southern and northern data (Table 1).

Douglas's global analysis⁴ included 21 sites, 8 of which were located on the east coast of North America. We have found that the average sea-level rate determined by correcting the 'raw' rates at the remaining 13 sites is 1.4 mm yr⁻¹ for a GIA correction based on our revised model, compared with 1.6 mm yr⁻¹ for the standard model (Table 1). The revised model thus yields a highly coherent global (GIA-corrected) tide-gauge record.

Previous studies which have considered only the standard GIA correction have been compelled to invoke a variety of mechanisms to explain the anomalous geographical trend in Fig. 1b. Variations in ocean circulation can influence sea level¹⁵, and it has been suggested that the anomalous trend may reflect the presence of the Gulf Stream⁴. However, the facts that the 'anomaly' is maintained for ~ 100 years and that the redirection of Gulf Stream flow occurs 3° – 5° south of the dominant sea-level gradient in Fig. 1b militate against this explanation⁴. Following earlier suggestions that sea-level variations on the east coast may be influenced by neotectonic processes^{16,17}, the residual tide-gauge record derived using a GIA model based on a lower-mantle viscosity of 2×10^{21} Pa s has been used by others² to identify possible sites of such activity. Although seismicity along the Atlantic seaboard

FIG. 3 Numerical predictions of the present-day rate of change of sea level (mm yr⁻¹; see colour scale) in the eastern United States due to GIA. The predictions were calculated using the ICE-3G deglaciation chronology⁸ and: a, the standard Earth model; b, as a, except that the lower-mantle viscosity has been increased to 4.7×10^{21} Pa s. The tide-gauge sites used in the study are indicated by crosses.



suggests the possibility of neotectonic activity, appreciable crustal deformations are ruled out by geological evidence¹⁸. The influence of ocean circulation and neotectonism on long-term (secular) sea-level rates on the US east coast has not been directly quantified, and both would have to be characterized by a very specific geographical variation to reconcile the 'raw' sea-level trends shown in Fig. 1a. The required variation is, as we have demonstrated, accurately obtained using a GIA correction based on the revised model.

The lower-mantle viscosity of the revised model (4.7×10^{21} Pa s) is consistent with several other recent inferences based on postglacial ('historical') relative sea level variations in southern Hudson Bay and/or northern Europe^{19,20}. Furthermore, models with a lower-mantle viscosity greater than the standard value, in combination with the ICE-3G deglaciation history⁸, have been found to yield misfits insignificantly different from those obtained using the standard model when a global database of Late Pleistocene sea-level histories is considered⁶. When histories from the east coast of North America only are considered, a model

similar to our revised model (lower-mantle viscosity 4×10^{21} Pa s) is in fact preferred⁶. The formal uncertainty in the revised lower-mantle viscosity ($\sim 0.2 \times 10^{21}$ Pa s when we propagate 3σ sea-level rate errors) is small, but may be somewhat misleading as it does not take into account deficiencies in the radial Earth model (for example, our simplistic description of the depth-dependence of viscosity) and ice-model errors^{12,21}. Non-negligible secular trends in either oceanographic or neotectonic sea-level signals, if they exist at all, will only significantly affect the lower-mantle viscosity estimate insofar as they can mimic peripheral bulge migration. Lateral heterogeneities in lithospheric strength can influence GIA predictions⁵, particularly at coastal margins^{22–25}, and may therefore alter our viscosity estimate. In this last case the main conclusion of this study would remain unaltered: the widely cited anomalous trend in GIA-corrected tide-gauge records from the North American east coast is entirely explicable in terms of inadequacies in the standard GIA model, and neither oceanographic nor neotectonic processes are required to explain the observations. □

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Phylogenetic position of the order Lagomorpha (rabbits, hares and allies)

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EVER since they have been classified as ruminants in the Old Testament (Leviticus 11:6, Deuteronomy 14:7) and equated with hyraxes in the vulgar Latin translation, rabbits and their relatives (order Lagomorpha) have frequently experienced radical changes in taxonomic rank. By using 91 orthologous protein sequences, we have attempted to answer the classical question "What, if anything, is a rabbit?"¹. Here we show that Lagomorpha is significantly more closely related to Primates and Scandentia (tree shrews) than it is to rodents. This newly determined phylogenetic position invalidates the superordinal taxon Glires (Lagomorpha + Rodentia), and indicates that the morphological 'synapomorphies' previously used to cluster rodents and lagomorphs into Glires^{2,3}, may actually represent symplesiomorphies or homoplasies that are of no phylogenetic value. This raises the possibility that the ancestral eutherian morphotype may have possessed many rodent-like morphological characters.

Linnaeus classified the lagomorphs as a family (Leporidae) within the order Rodentia. Subsequently, Lagomorpha has been

elevated twice in taxonomic rank, first to the rank of suborder (Duplicidentata) within Rodentia, and then to the rank of order¹. The monophyletic status of Lagomorpha is undisputed, and has been supported most recently by molecular data^{4,5}. The evolutionary affinities of Lagomorpha to the other eutherian orders, however, remain controversial, and on morphological grounds the order has been variably placed as a sister group of Rodentia within cohort Glires⁶, a sister group of Macroscelidea (elephant shrews) within cohort Anagalidia⁷, or in an indeterminate position relative to the other eutherian orders¹. Previous molecular attempts to identify the phylogenetic position of rabbits within the eutherian tree have cast serious doubt on the validity of Glires, but have failed to provide strong positive evidence concerning the superordinal affinities of Lagomorpha^{8–11}. Here we have used orthologous protein sequences to assess the evolutionary relationships of Lagomorpha to other eutherian taxa.

We analysed 88 protein sequences for which data from Sciurornathi, Lagomorpha, Primates and an outgroup species are available (Table 1). With three ingroup taxa and an outgroup, there are three possible phylogenetic trees (Fig. 1), of which the traditional phylogeny is shown in Fig. 1a. All reconstruction methods result in the topology given in Fig. 1b, that is, primates are phylogenetically closer to lagomorphs than either taxa are to sciurornaths. The internal branch, although short, was significantly different from 0 ($P < 0.001$), and was supported in the neighbour-joining analysis by all 1,000 bootstrap replicates. In parsimony analysis the length of the tree in Fig. 1b is 10,328 amino acid replacements, shorter by 121 replacements than the traditional tree (Fig. 1a). Interestingly, even the second unorthodox tree (Fig. 1c) was shorter by 47 replacements than the traditional tree. The log-likelihood bootstrap probability of Fig. 1b tree is 0.71, as opposed to only 0.15 for the Fig. 1a tree. With all three methods of phylogenetic reconstruction, identical results were

TABLE 1 Neighbour-joining trees for Lagomorpha, Primates, an outgroup and a test eutherian taxon

Test eutherian taxon	Number of proteins	Number of aligned amino-acid sites	Sister taxon of Primates in the four-taxon analysis	Bootstrap value (%)*
Sciurognathi	88	24,865	Lagomorpha	100
Hystricognathi	21	3,528	Lagomorpha	98
Artiodactyla + Cetacea	62	14,492	Lagomorpha	99
Carnivora	32	7,057	Lagomorpha	100
Edentata	4	682	Lagomorpha	99
Pholidota	1	172	Lagomorpha	100
Sirenia	3	459	Lagomorpha	95
Tubulidentata	2	325	Lagomorpha	95
Macroscelidea	1	172	Lagomorpha	100
Hyracoidea	3	459	Lagomorpha	85
Insectivora	3	440	Lagomorpha	86
Proboscidea	7	1,118	Lagomorpha	88
Chiroptera	9	1,427	Lagomorpha	63
Perissodactyla	23	4,114	Lagomorpha	66
Dermoptera	3	629	Dermoptera	55
Scandentia	7	1,151	Outgroup	62

We have collected 91 orthologous protein sequence sets for which orthologous sequence data exist for lagomorphs and at least two other eutherian orders and an outgroup (a marsupial, a monotreme, a bird or a reptile) using the HOVERGEN¹³ and PIR¹⁴ databases. The list of proteins is available on World Wide Web at <http://acnuc.univ-lyon1.fr/data/lagomorpha.phylo.html>. Whenever possible, a mammalian outgroup was preferred to a reptilian or an avian one. Orthology, as opposed to paralogy, was ascertained by either consulting the primary literature or by using HOVERGEN to separate gene families into orthologous sets. It is expected that those few paralogous sequences that may have remained in our database will merely increase the random phylogenetic noise, rather than yield consistent erroneous phylogenies. The orthologous sets were aligned by using the CLUSTAL W program¹⁵. Ambiguous parts in the alignments (as judged by visual inspection), as well as gaps, have been removed from further analysis. If a certain eutherian order was represented in a sequence set by two or more species, we constructed a neighbour-joining phylogenetic tree, and selected the sequences with the shortest branch length to represent the order in question. Implicit in this procedure is the assumption that each taxon dealt with in this study is monophyletic, and therefore valid. Because of possible paraphyly of Rodentia^{16,17}, its two constituent suborders, Sciurognathi (including mice and squirrels) and Hystricognathi (including guinea pigs), have been treated as separate taxa. The results are, however, unaffected by the phyletic status of Rodentia. On grounds of well-established phylogenetic intimacy, the orders Cetacea and Artiodactyla were treated as a single taxon¹⁸. The eutherian taxa used were: Lagomorpha, Primates, Artiodactyla + Cetacea, Carnivora, Chiroptera, Dermoptera, Edentata, Hyracoidea, Insectivora, Perissodactyla, Macroscelidea, Pholidota, Proboscidea, Scandentia, Sirenia, Tubulidentata, Sciurognathi, and Hystricognathi. To maximize the use of the molecular data, we dealt with four taxa at a time.

* Based on 1000 replicates.

obtained by using the second suborder of rodents, Hystricognathi. The length of the internal branch is significantly different from 0, and was supported by 980 out of 1,000 bootstrap replicates. In the parsimony analysis, the trees in Fig. 1b and 1c were found to be shorter by 37 and 3 amino acid replacements, respectively, than that in Fig. 1a. The bootstrap probability of the Fig. 1b tree under the maximum-likelihood method is 0.90 as opposed to 0.09 for the tree in Fig. 1a. These results contradict the taxonomic validity of Glires.

This raises the question of whether primates are the closest relatives of the lagomorphs or whether other eutherian orders are closer to Lagomorpha than are the primates. Each taxon in our

compilation was phylogenetically tested against Lagomorpha, Primates and an outgroup. With the exception of Dermoptera and Scandentia, all other orders emerged as outgroups of a Lagomorpha–Primates clade. Bootstrap values were larger than 85% for 12 orders (Table 1). The inferred close phylogenetic affinity between lagomorphs and primates to the exclusion of Macroscelidea, although based on a single sequence, argues against a phylogenetic proximity between rabbits and elephant shrews. Similarly, on the basis of two amino acid sequences, Macroscelidea was shown to be unrelated to either suborder of rodents (data not shown). Indeed, published data suggests that elephant shrews are related to the paenungulates¹², a conclusion

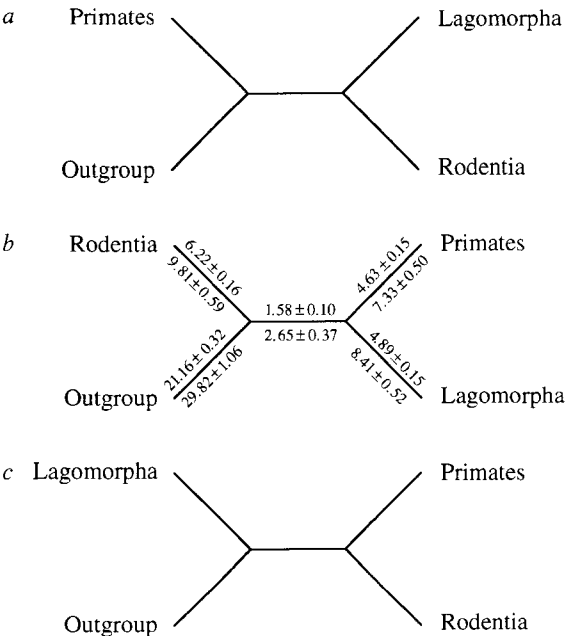


FIG. 1 Three possible phylogenetic trees for Lagomorpha, Primates, a rodent, and an outgroup. a, The tree represents traditional morphological taxonomy. b, On the basis of molecular data, this tree emerges as the most-supported phylogenetic hypothesis. Maximum-likelihood branch lengths in units of amino-acid replacements per 100 amino-acid sites ($\pm$ s.e.) are given. Upper values, Sciurognathi (88 proteins); lower values, Hystricognathi (21 proteins). c, Unorthodox tree.

METHODS. Phylogenetic trees were constructed by using three reconstruction methods: neighbour-joining¹⁹ by means of the CLUSTAL W program¹⁵, maximum-parsimony with the PROTPARS program in the PHYLIP package²⁰, and maximum-likelihood with PROTML²¹. Genetic distances between amino acid sequences were computed by correcting for multiple hits²². Reliability of internal branches of neighbour-joining trees has been ascertained by bootstrap analysis²³ and Li's test²⁴. To evaluate the extent to which a maximum-likelihood tree is a significantly better representation of the true tree than the alternative possible trees, the relative bootstrap probabilities of all the possible trees were estimated²⁵.

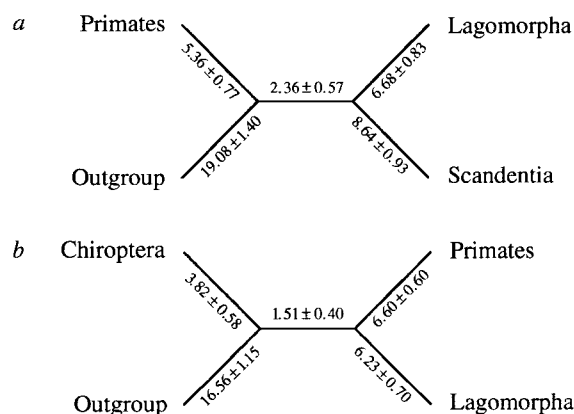


FIG. 2 Maximum-likelihood trees for Lagomorpha, Primates, an outgroup, and Scandentia (a) or Chiroptera (b). Maximum-likelihood branch lengths ($\pm$ s.e.) are given in units of amino-acid replacements per 100 amino-acid sites. a, The tree is based on 7 orthologous protein sequences (1,157 amino acids). b, This tree is based 9 proteins (1,433 amino acids).

strongly supported by analyses of complete mitochondrial DNA sequences (Ú. Arnason, personal communication). Therefore, cohort Anagalidia or the positioning of Macroscelidea as an immediate outgroup to a monophyletic Glires are invalidated by the molecular data. We can also reject the monophyly of Glires by noting that four taxa other than Primates, these being Artiodactyla + Cetacea, Carnivora, Scandentia, and Dermoptera, are significantly closer phylogenetically to Lagomorpha than are the rodents (data not shown).

Finally, we tried to pinpoint more accurately the phylogenetic position of Lagomorpha. Three orders are thought on morphological grounds to be closely related to the primates: Scandentia, Dermoptera and Chiroptera⁶, which together are included within a superordinal taxon called Archonta. Scandentia is considered particularly close phylogenetically to Primates, and the two orders are joined in morphological systems of classification within a superorder called Primatomorpha, whereas Chiroptera and Dermoptera form a sister superordinal taxon called Volitanti⁶. In a maximum-likelihood analysis, Scandentia was found to cluster with Lagomorpha to the exclusion of Primates and the outgroup (Fig. 2a), with a log-likelihood bootstrap probability of 0.80, as opposed to 0.17 for the tree in which Primatomorpha is monophyletic. Primates clustered with Lagomorpha to the exclusion of Chiroptera, a phylogenetic arrangement supported by a log-likelihood bootstrap probability of 0.74, as opposed to 0.07 for the tree in which Archonta is monophyletic (Fig. 2b). These results add to the accumulating molecular evidence against the monophyly of Archonta¹⁰. The phylogenetic position of Dermoptera relative to Primates and Lagomorpha could not be resolved with the available data (3 proteins, 629 amino acids). Pending a significant increase in the database for dermopterans and scandentians, we conclude tentatively that Scandentia and Lagomorpha are sister orders.

About a dozen anatomical 'synapomorphies' form the morphological basis for the clustering of Rodentia and Lagomorpha³. Some of them, such as 'orbitosphenoid relatively large' or 'incisive foramina enlarged' are so vaguely defined as to defy objective analysis. Other traits, such as a 'blastocyst attachment invasive', also typify other eutherian orders, but these conditions are usually brushed aside as being 'derived secondarily'. Many character-state resemblances between rodents and lagomorphs, for example, the absence of canines, are related to specializations due to the evolution of gnawing in both orders, and as such may be subject to rampant parallelism¹. Finally, the polarities of morphological character states as either 'derived' or 'primitive' are decided on the basis of comparisons with a eutherian 'morphotype', an artificial construct based on assumptions about hypothetical

ancestral taxa. Errors in polarity identification may alter the phylogenetic tree considerably. Our molecular results indicate that a reassessment of the morphological evidence is needed. One simple and intriguing possible resolution of the conflict between morphological and molecular data is to assume that many morphological 'synapomorphies' used in support of Glires are actually ancestral character states that have been retained in some mammalian orders but were lost in others. If this reversal of character-state polarity proves valid, then the ancestral eutherian morphotype may have resembled a rodent species much more closely than is currently recognized in the morphopaleontological literature. □

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Molecular basis of sun-induced premature skin ageing and retinoid antagonism

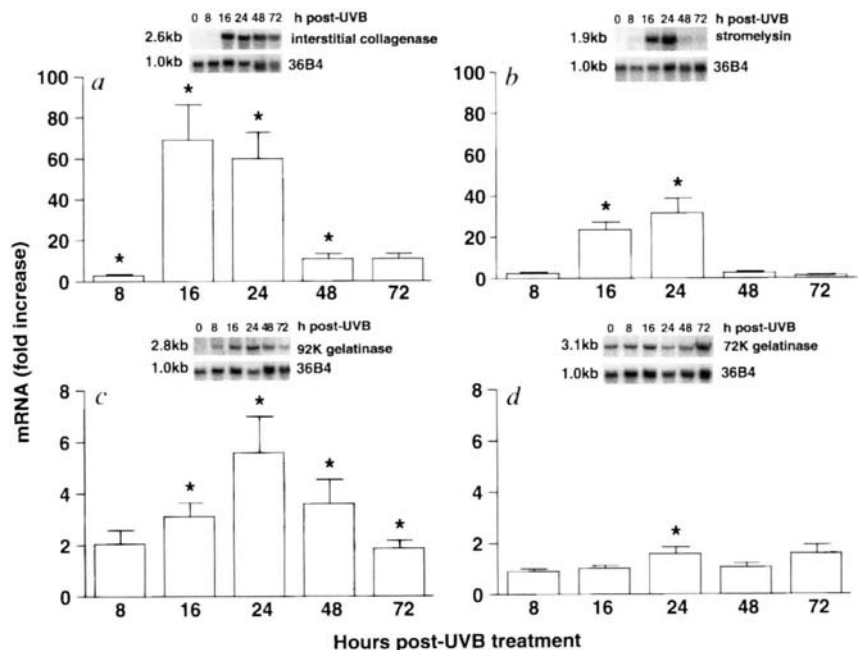
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DAMAGE to skin collagen and elastin (extracellular matrix) is the hallmark of long-term exposure to solar ultraviolet irradiation^{1–3}, and is believed to be responsible for the wrinkled appearance of sun-exposed skin^{4,5}. We report here that matrix-degrading metalloproteinase messenger RNAs, proteins and activities are induced in human skin *in vivo* within hours of exposure to ultraviolet-B irradiation (UVB). Induction of metalloproteinase proteins and activities occurred at UVB doses well below those that cause skin reddening. Within minutes, low-dose UVB

FIG. 1 UVB induces interstitial collagenase, 92K gelatinase, and stromelysin I mRNA, but not 72K gelatinase mRNA, in human skin *in vivo*. Adult buttocks were irradiated with 2 MED UVB. Irradiated sites and adjacent non-irradiated sites were removed at indicated times following irradiation. Tissue was snap-frozen, and total RNA isolated and analysed by northern blot²⁶. Band intensities were quantified using a PhosphorImager. Values for metalloproteinase transcripts were normalized to those for control gene 36B4. Results are means $\pm$ s.e.m. ($n = 6$ for 8, 16, 48 and 72 h, and $n = 17$ for no-UVB control and 24 h), and are presented as fold increase of normalized values relative to non-irradiated skin. Insets show representative bands at indicated times after irradiation. *a*, Interstitial collagenase; *b*, stromelysin I; *c*, 92K gelatinase; *d*, 72K gelatinase. * $P < 0.05$ compared with no UVB control.

METHODS. Subjects were irradiated with an Ultra-lite Panelite lamp containing four F36T12 ERE-VHO UVB tubes. Irradiation intensity was monitored with an IL443 phototherapy radiometer and a SED240/UVB/W photodetector (International Light, Newbury, MA). UVB output, measured 48 cm from the source, was 0.5 mW cm^{-2} . Subjects were adult Caucasians (approximately equal numbers of males and females) with light to mild pigmentation. Minimal erythema dose (MED) UVB for each subject was determined 24 hours post irradiation. 1MED for all subjects ranged from $30\text{--}50 \text{ mJ cm}^{-2}$. For each subject, skin was removed by keratome²⁶ from four sites (one non-irradiated and three irradiated). Thus, bands displayed in figures are composites from several individuals. All subjects provided informed written consent, and procedures involving humans were approved



by the University of Michigan Institutional Review Board. Northern analysis of UVB-treated skin with a stromelysin I-specific probe yielded results identical to those obtained with full length stromelysin I probe (*b*), whereas hybridization with a stromelysin II-specific probe yielded no signal. We conclude that UVB induces predominantly stromelysin I in human skin. Data were analysed by two-tailed paired *t*-test.

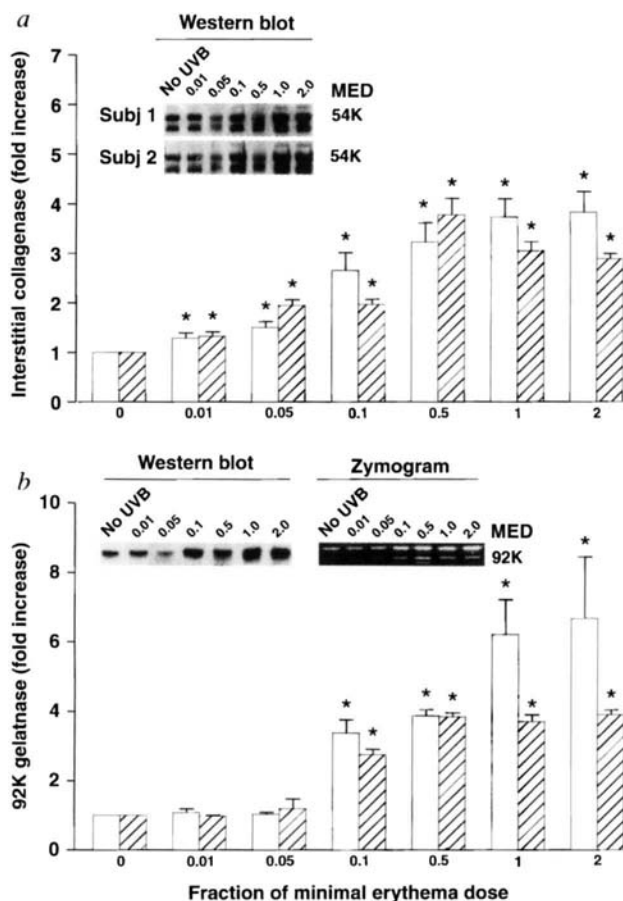


FIG. 2 Low-dose UVB induces interstitial collagenase and 92K gelatinase proteins and activities in human skin *in vivo*. Adult subjects were exposed to the indicated doses of UVB as described in Fig. 1 legend. Full thickness skin (6 mm cylinders) was obtained 24 hours after irradiation. *a*, Interstitial collagenase protein (□), determined by western blot, and activity (▨). The inset shows representative western blots from two subjects. The larger 54K band is intact interstitial collagenase, and the smaller 45K band is the proteolytically processed activated form of the molecule¹¹. Note both forms of interstitial collagenase are elevated following UVB irradiation. *b*, 92K gelatinase protein (□), determined by western blot, and activity (▨). The inset shows a representative western blot (left panel) and a representative zymogram (right panel). Multiple bands on the zymogram are proteolytically processed active forms of the enzyme, determined by immunoblot of the zymogram (data not shown). Note unprocessed and processed forms of 92K gelatinase are induced by UVB. Band intensities were quantified by laser densitometry. Results are means $\pm$ s.e.m., $n = 10$; * $P < 0.025$ vs no UVB control.

METHODS. Skin from irradiated and adjacent non-irradiated sites was homogenized in 20 mM Tris-HCl (pH 7.6), 5 mM CaCl_2 , and centrifuged at $3,000g$ for 10 min. Supernatants were used to measure interstitial collagenase and 92K gelatinase proteins by western blot (100 μg per lane), using chemiluminescence detection, and activities by hydrolysis of [^3H]fibrillar collagen²⁷ (100 μg per assay) and gelatin zymography²⁸ (20 μg per assay), respectively. Antibodies against interstitial collagenase²⁹ and 92K gelatinase³⁰ have been described.

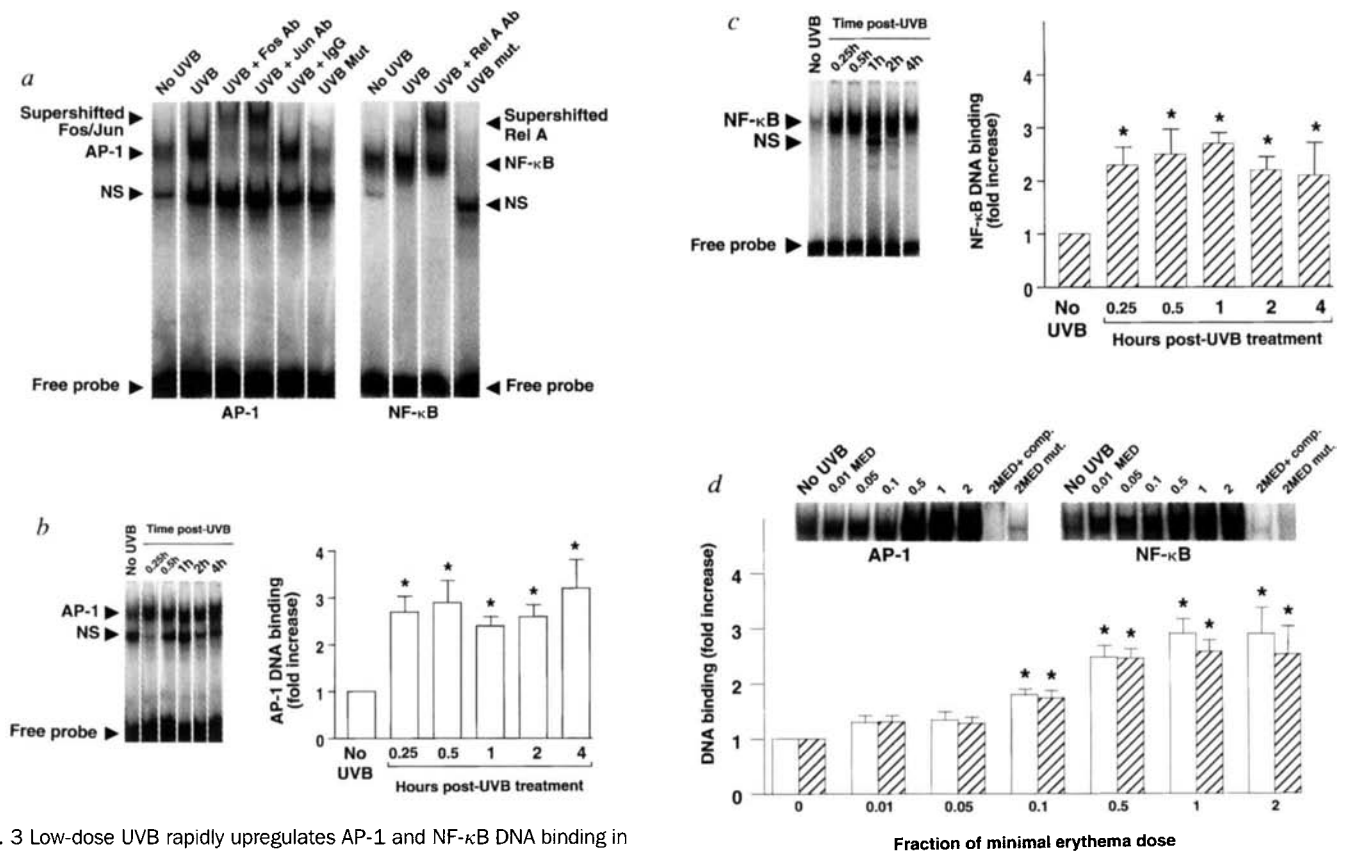


FIG. 3 Low-dose UVB rapidly upregulates AP-1 and NF- κ B DNA binding in human skin *in vivo*. Subjects were irradiated with UVB as described in Fig. 1 legend. At the indicated times after irradiation, tissue was taken by keratome and immediately processed to obtain nuclear extracts. AP-1 and NF- κ B binding to double-stranded DNA probes were measured by electrophoretic mobility-shift assays. **a**, AP-1 and NF- κ B DNA binding in non-irradiated and irradiated (4 h after 2 MED UVB) skin. Supershifts using antibodies against Jun and Fos family members and p65/Rel A demonstrated the presence of these proteins in AP-1 and NF- κ B retarded complexes, respectively. Reduced binding to mutant probes demonstrates specificity of AP-1 and NF- κ B retarded complexes. NS, nonspecific complex. **b**, Time course of induction of AP-1 DNA binding by 2 MED UVB. Results are means $\pm$ s.e.m., $n = 9$. * $P < 0.01$ compared with no UVB control. **c**, Time course of induction of NF- κ B DNA binding by 2 MED UVB. Results are means $\pm$ s.e.m., $n = 9$. * $P < 0.01$ compared with no UVB control. **d**, UVB dose-dependence of induction of AP-1 (white bars) and NF- κ B (striped bars). DNA binding was measured 30 min after irradiation. * $P < 0.02$ versus no UVB control. Also shown are representa-

tive AP-1 and NF- κ B retarded complexes. +comp., Addition of 100-fold excess unlabelled probe. mut., Mutated 32 P-labelled probe. Intensities of retarded complexes were quantified by PhosphorImager. Results are means $\pm$ s.e.m., $n = 9$.

METHODS. Nuclear extracts were prepared from skin biopsies as described²¹. Biopsies (~200 mg wet weight) containing 10^8 cells yielded 500 μ g nuclear extract protein, on average. Electrophoretic mobility-shift assays (8 μ g nuclear extract protein) were performed using 32 P-labelled DNA probes containing AP-1 and NF- κ B consensus and mutated DNA-binding sequences, as described²¹. SP-1 DNA binding was also determined, and found to not vary significantly between non-irradiated and irradiated samples (data not shown). Antibodies for supershifts were obtained from Santa Cruz Biotechnology. Jun and Fos antibodies had broad reactivity to all Jun and Fos family members, respectively. NF- κ B antibody was specific for p65/Rel A.

upregulated the transcription factors AP-1 and NF- κ B, which are known to be stimulators of metalloproteinase genes^{6,7}. All-trans retinoic acid, which transrepresses AP-1 (ref. 8), applied before irradiation with UVB, substantially reduced AP-1 and metalloproteinase induction. We propose that elevated metalloproteinases, resulting from activation of AP-1 and NF- κ B by low-dose solar irradiation, degrade collagen and elastin in skin. Such damage, if imperfectly repaired, would result in solar scars, which through accumulation from a lifetime of repeated low-dose sunlight exposure could cause premature skin ageing (photoageing).

We determined the time course of changes in interstitial collagenase, stromelysin I, gelatinase of relative molecular mass 92,000 (M_r 92K), and 72K gelatinase mRNA levels, following UVB exposure. These four metalloproteinases together possess the capacity to degrade collagenous and non-collagenous molecules in extracellular matrix of skin⁹⁻¹². Human subjects were irradiated on buttock skin with twice the UVB dose required to cause barely perceptible skin reddening (minimal erythema dose, MED). Induction of interstitial collagenase (Fig. 1a), stromelysin I (Fig. 1b), and 92K gelatinase (Fig. 1c) mRNAs was maximal (6–60-fold) within 16 to 24 hours, and returned to near

baseline within 48 to 72 hours. 72K gelatinase mRNA was detectable but was only elevated 1.6-fold at 24 hours post-UVB (Fig. 1d).

Time courses for induction of interstitial collagenase and 92K gelatinase proteins and activities by 2 MED UVB paralleled those observed for their mRNAs (data not shown). Also consistent with northern analysis, neither 72K gelatinase protein nor enzyme activity was induced (data not shown), and thus served as an internal control. Induction of interstitial collagenase (Fig. 2a) and 92K gelatinase (Fig. 2b) proteins and activities by UVB was dose-dependent, and for both metalloproteinases, changes in protein content and enzyme activity mirrored each other. Interstitial collagenase was induced by all doses of UVB tested (0.01–2 MED), whereas 92K gelatinase was induced by ≥ 0.1 MED. Induction of both interstitial collagenase and 92K gelatinase was maximal at 1 MED and approximately half-maximal at 0.1 MED. This dose of UVB is equivalent to 2–3 min solar irradiation on a summer day, which causes no perceptible skin reddening. Skin injuries such as blisters and wounds induce interstitial collagenase, 92K gelatinase, and stromelysin in cells within or adjacent to extracellular matrix, where their substrates are localized^{9,10}. It is likely therefore that metalloproteinases induced

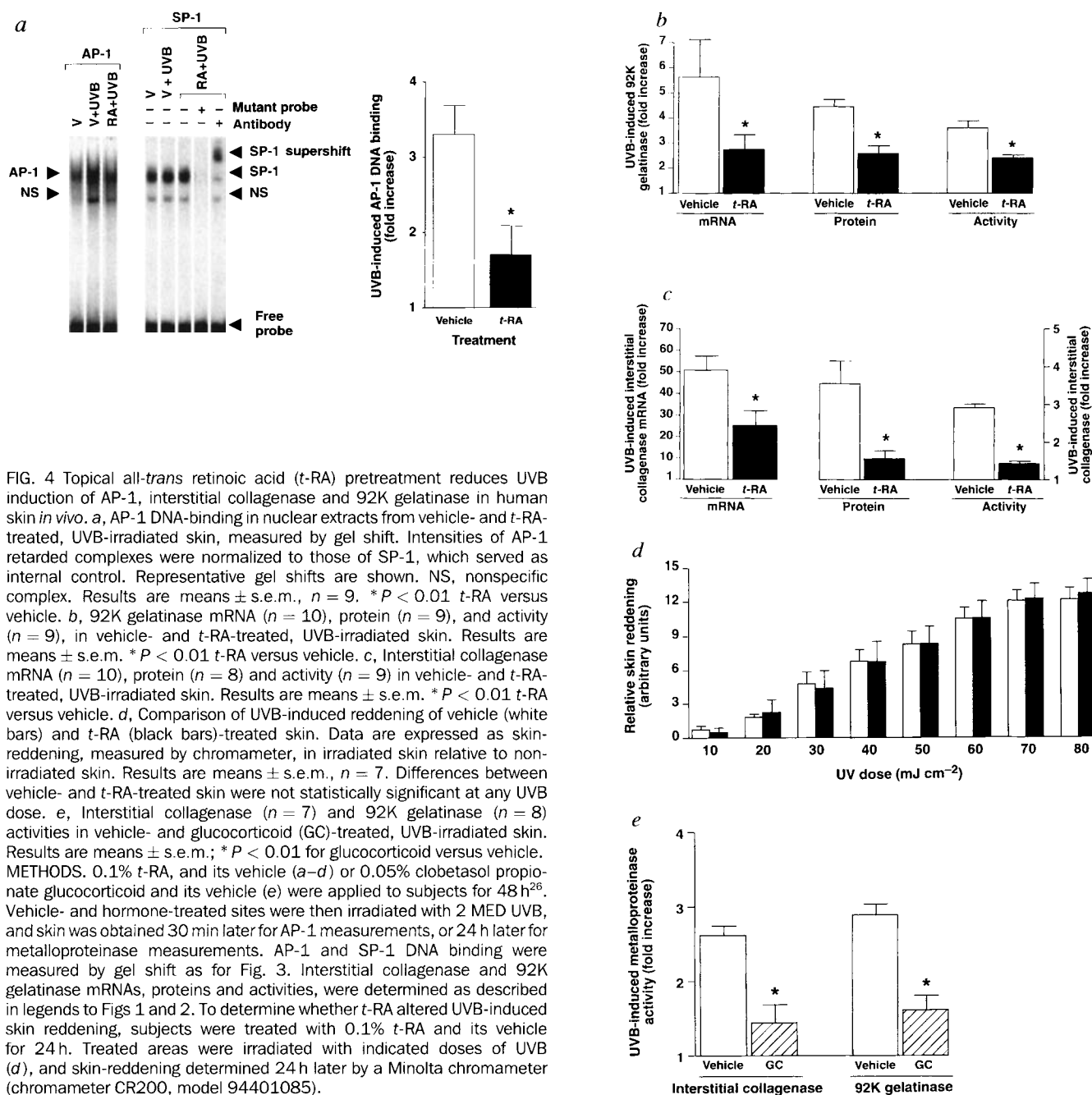


FIG. 4 Topical all-trans retinoic acid (t-RA) pretreatment reduces UVB induction of AP-1, interstitial collagenase and 92K gelatinase in human skin *in vivo*. **a**, AP-1 DNA-binding in nuclear extracts from vehicle- and t-RA-treated, UVB-irradiated skin, measured by gel shift. Intensities of AP-1 retarded complexes were normalized to those of SP-1, which served as internal control. Representative gel shifts are shown. NS, nonspecific complex. Results are means $\pm$ s.e.m., $n = 9$. * $P < 0.01$ t-RA versus vehicle. **b**, 92K gelatinase mRNA ($n = 10$), protein ($n = 9$), and activity ($n = 9$), in vehicle- and t-RA-treated, UVB-irradiated skin. Results are means $\pm$ s.e.m. * $P < 0.01$ t-RA versus vehicle. **c**, Interstitial collagenase mRNA ($n = 10$), protein ($n = 8$) and activity ($n = 9$) in vehicle- and t-RA-treated, UVB-irradiated skin. Results are means $\pm$ s.e.m. * $P < 0.01$ t-RA versus vehicle. **d**, Comparison of UVB-induced reddening of vehicle (white bars) and t-RA (black bars)-treated skin. Data are expressed as skin-reddening, measured by chromameter, in irradiated skin relative to non-irradiated skin. Results are means $\pm$ s.e.m., $n = 7$. Differences between vehicle- and t-RA-treated skin were not statistically significant at any UVB dose. **e**, Interstitial collagenase ($n = 7$) and 92K gelatinase ($n = 8$) activities in vehicle- and glucocorticoid (GC)-treated, UVB-irradiated skin. Results are means $\pm$ s.e.m.; * $P < 0.01$ for glucocorticoid versus vehicle. **METHODS**. 0.1% t-RA, and its vehicle (**a–d**) or 0.05% clobetasol propionate glucocorticoid and its vehicle (**e**) were applied to subjects for 48 h²⁶. Vehicle- and hormone-treated sites were then irradiated with 2 MED UVB, and skin was obtained 30 min later for AP-1 measurements, or 24 h later for metalloproteinase measurements. AP-1 and SP-1 DNA binding were measured by gel shift as for Fig. 3. Interstitial collagenase and 92K gelatinase mRNAs, proteins and activities, were determined as described in legends to Figs 1 and 2. To determine whether t-RA altered UVB-induced skin reddening, subjects were treated with 0.1% t-RA and its vehicle for 24 h. Treated areas were irradiated with indicated doses of UVB (**d**), and skin-reddening determined 24 h later by a Minolta chromameter (chromameter CR200, model 94401085).

by UVB are localized to act on their extracellular matrix substrates, as occurs with other types of skin injury.

Transcription factors AP-1, composed of Jun and Fos proteins, and NF- κ B, composed of Rel family members, are activated by a variety of extracellular stimuli, including short-wavelength UV (UVC)¹³. *In vitro*, stimulation of interstitial collagenase¹⁴, 92K gelatinase⁷, and stromelysin I (ref. 15) gene transcription by extracellular mediators is dependent upon activation and binding of AP-1 to specific sequences in their promoters. 92K-gelatinase gene transcription is also positively regulated by NF- κ B (ref. 7). We therefore determined whether UVB irradiation regulates AP-1 and NF- κ B in human skin *in vivo*.

Two MED UVB upregulated AP-1 and NF- κ B DNA binding (Fig. 3a). Binding of both transcription factors to their DNA response elements was specific, as demonstrated by loss of retarded complexes with mutated labelled probes (Fig. 3a). Antibody supershifts demonstrated that the specific AP-1 and NF- κ B retarded complexes observed with extracts from UVB-irradiated

skin contained Jun and Fos proteins, and Rel A protein, respectively (Fig. 3a). Induction of AP-1 (Fig. 3b) and NF- κ B (Fig. 3c) by 2 MED UVB occurred within 15 min and remained elevated for at least 24 hours (data not shown). In contrast DNA binding of transcription factor SP-1, as an internal control, did not vary significantly between non-irradiated and irradiated samples at any time (data not shown).

Half-maximal induction of AP-1 and NF- κ B, measured 30 min after irradiation, occurred at ~ 0.1 MED and maximal induction at 1 MED (Fig. 3d). The UVB dose dependencies for induction of AP-1 and NF- κ B closely matched those observed for induction of interstitial collagenase and 92K gelatinase (Fig. 2), consistent with participation of AP-1 and NF- κ B in UVB-induced increases in these metalloproteinases. Of note is that the 92K gelatinase gene is not induced by UVB, and does not contain either AP-1 or NF- κ B response elements in its promoter¹⁶.

Several members of the nuclear receptor superfamily, including retinoic acid^{18,17} and glucocorticoid^{18,19} receptors, are capable of

transrepressing AP-1 activity. Transrepression is ligand-dependent, and apparently involves protein-protein interactions between hormone receptors and Jun or Fos²⁰. These interactions result in complexes that lack transactivation capacity for either AP-1 or hormone-response elements²⁰. Pretreatment of skin, which expresses retinoic acid receptors²¹, with all-*trans* retinoic acid (*t*-RA) reduced UVB-induced AP-1 DNA binding by 70% (Fig. 4a). *t*-RA pretreatment also reduced UVB-induced 92K gelatinase (Fig. 4b) and interstitial collagenase (Fig. 4c) mRNAs, proteins and activities by 50–80%. 72K gelatinase mRNA, protein and activity, which were not induced by UVB, were also not altered by *t*-RA (data not shown). Although *t*-RA absorption overlaps the UVB range (*t*-RA, λ_{max} 351 nm), *t*-RA did not reduce UVB-induced skin reddening (Fig. 4d) (that is, *t*-RA is not a sunscreen, at least for the chromophore(s) responsible for skin reddening), indicating that observed reductions in AP-1, interstitial collagenase and 92K gelatinase induction were specific, rather than being due to absorption of UVB by *t*-RA. Furthermore, glucocorticoid (λ_{max} 237 nm) pretreatment, which like *t*-RA has AP-1 transrepression activity, also reduced UVB-induced interstitial collagenase and 92K gelatinase activities to extents similar to *t*-RA (Fig. 4e). Our data are consistent with *t*-RA transrepression of AP-1-mediated induction of interstitial collagenase and 92K gelatinase, although limitations of *in vivo* experimentation mean that other mechanisms cannot be excluded.

Collagen and elastin provide structural integrity to skin²². Induction by sunlight of metalloproteinases that degrade collagen and elastin in skin^{12,23} may therefore be the primary mechanism mediating cutaneous photoageing. Based on our data, it is conceivable that *t*-RA, which improves photoageing once it has occurred²⁴, and other molecules that inhibit UVB-induced metalloproteinases²⁵, may reduce sun-induced collagen degradation and prevent photoageing in human skin. □

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Normal host prion protein necessary for scrapie-induced neurotoxicity

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ACCUMULATION of the prion protein PrP^{Sc}, a pathological and protease-resistant isoform of the normal host protein PrP^C, is a feature of prion disease such as scrapie^{1,2}. It is still unknown whether scrapie pathology comes about by neurotoxicity of PrP^{Sc}, acute depletion of PrP^C, or some other mechanism. Here we investigate this question by grafting neural tissue overexpressing PrP^C into the brain of PrP-deficient mice which are scrapie-resistant and do not propagate infectivity^{3–5}. After intracerebral inoculation with scrapie prions, the grafts accumulated high levels of PrP^{Sc} and infectivity and developed the severe histopathological changes characteristic of scrapie. Moreover, substantial amounts of graft-derived PrP^{Sc} migrated into the host brain. Even 16 months after inoculation no pathological changes were seen in PrP-deficient tissue, not even in the immediate

vicinity of the grafts. Therefore, in addition to being resistant to scrapie infection, brain tissue devoid of PrP^C is not damaged by exogenous PrP^{Sc}.

We exposed brain tissue of PrP-deficient (*Pm-p*^{0/0}) mice to a continuous source of PrP^{Sc} by grafting embryonic telencephalic tissue from transgenic mice overexpressing PrP into the caudoputamen or lateral ventricles of *Pm-p*^{0/0} mice and inoculating it with scrapie prions. The donors, transgenic *tga20* mice⁶, contain an array of 30–50 gene copies encoding PrP, overexpress PrP^C 5–8 fold, and show incubation times of around 60 days as compared to 160 days for CD-1 wild-type mice. In the terminal stage of scrapie both mouse strains exhibit similar prion titres whereas PrP^{Sc} levels in *tga20* are 25–50% lower than in CD-1 brain⁶. Twenty-six *Pm-p*^{0/0} animals engrafted with *tga20* tissue, three with wild-type and seven with *Pm-p*^{0/0} tissue, were inoculated intracerebrally with scrapie prions. All mice remained free of scrapie symptoms for at least 70 weeks; this exceeds the survival time of scrapie-infected *tga20* mice sevenfold. However, *tga20* and wild-type grafts developed characteristic histopathological features of scrapie 70 and 160 days after inoculation (p.i.), respectively, reflecting the incubation time of scrapie in the respective donor animals^{4,6}. Infection of wild-type mice harbouring *tga20* grafts (*n* = 4) induced scrapie pathology in both graft and host brain, indicating efficient spread of prions from host to graft (Fig. 1a–c). Uninfected (*n* = 9) or mock-infected (*n* = 7) *tga20* grafts occasionally showed mild gliosis but never spongiosis (Fig. 1d–f). Therefore, high expression of PrP by itself did not induce neurodegeneration in grafts.

Early stages of the disease in the graft (70–140 days p.i.) were characterized by spongiosis, gliosis and reduced synaptophysin immunoreactivity (Fig. 1g–i), which we found in terminally sick *tga20* mice, whereas intermediate-stage grafts (140–280 days p.i.) showed *status spongiosus* with dramatic ballooning and loss of neurons, gliosis and stripping of neuronal processes (Fig. 1j–l). At

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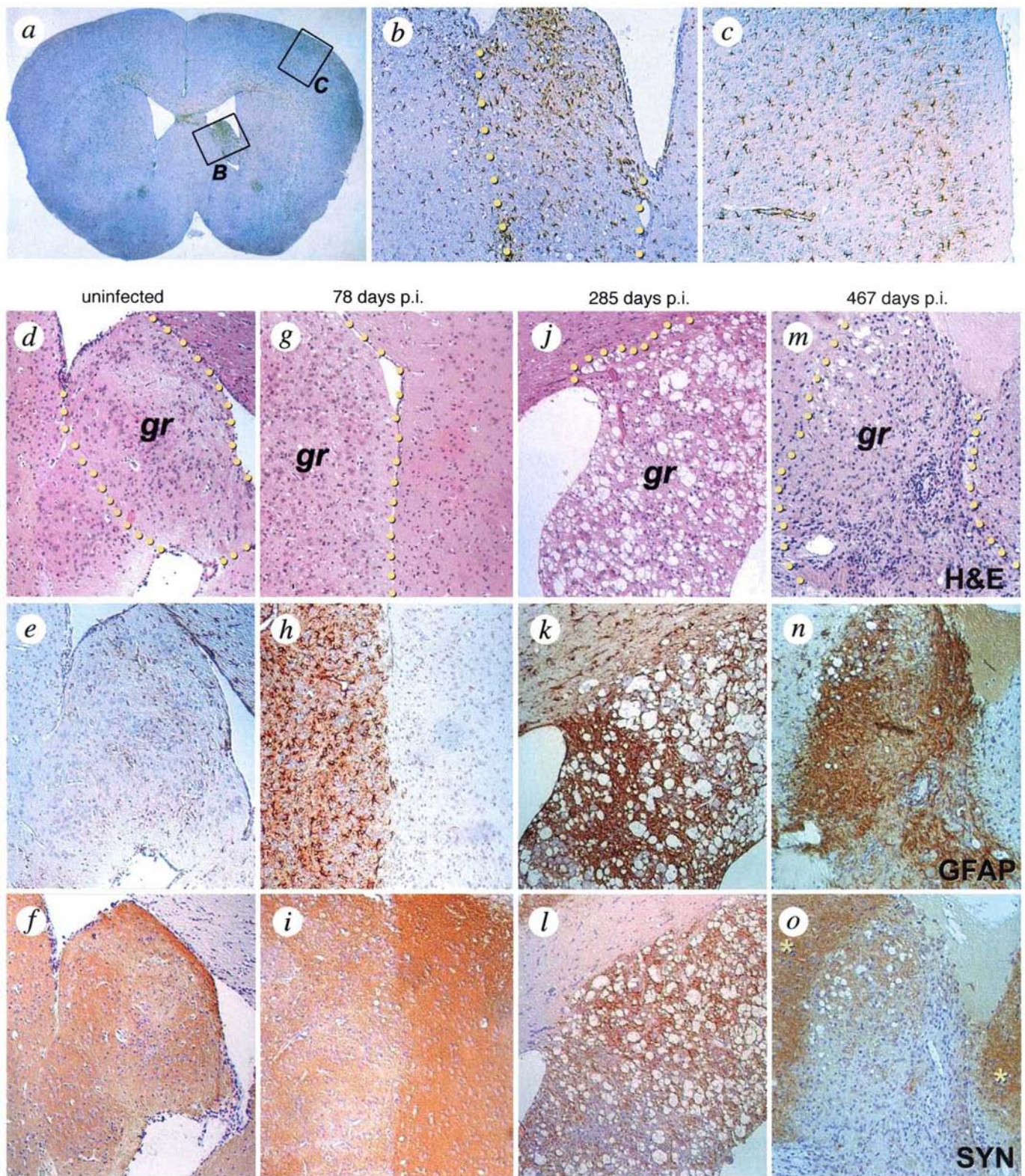


FIG. 1 PrP^C-expressing grafts in *Prn-p*^{+/+} (a–c) and *Prn-p*^{0/0} brains (d–o) at different times after inoculation with scrapie-infected brain extract. a, *tga20* graft (asterisk) in a terminally sick *Prn-p*^{+/+} mouse: pathology is more pronounced in the graft (b, enclosed by dotted line) than in host cortex (c). d, Control graft 235 days after mock inoculation with non-infectious brain extract⁴, showing fully differentiated neuroectodermal tissue staining weakly with glial fibrillary acidic protein (GFAP) (e) and strongly with synaptophysin (f). Seventy-eight days p.i., grafts developed significant spongiosis (g), marked astrogliosis with strong GFAP immunoreactivity (h) and reduction of synaptophysin immunoreactivity (i). The host brain appears histologically normal 285 days p.i. (j); extreme spongiform changes (vacuole diameter > 40 µm) and GFAP-positive reactive astrocytes are apparent (k). l, The coarse pattern of synaptophysin immunostaining indicates

neuronal damage. m, 467 days p.i., nearly complete loss of neuropil results in hypercellularity. The graft consists mainly of clustered GFAP-positive astrocytes (n); lack of synaptophysin immunoreactivity indicates virtually complete loss of neurons (o). Even at this late stage, there is no gliosis, spongiosis or reduced synaptophysin immunoreactivity visible in host tissue (asterisks) immediately adjacent to the graft, indicating that no neuronal damage had occurred.

METHODS. Brain cell suspensions (5–10 × 10⁶ cells) from heterozygous or homozygous *tga20* (ref. 6), *Prn-p*^{0/0} or wild-type embryos (E12.5) were stereotactically injected as described⁸. *Prn-p*^{0/0} and wild-type mice grafted with *tga20* tissue were inoculated intracerebrally with 10⁷ ID₅₀ units of RML mouse prions⁴ 48 h or 25 days after grafting.

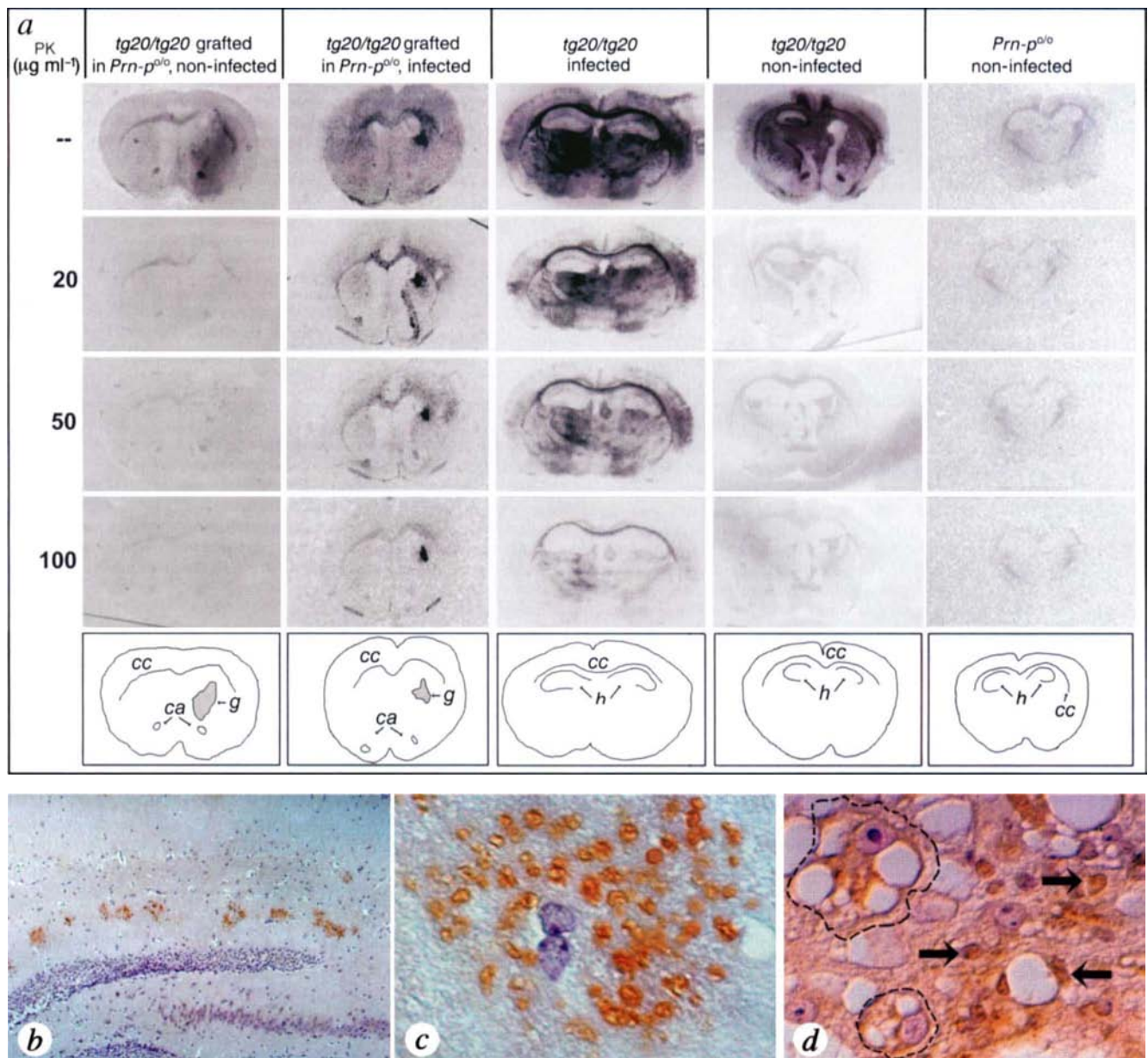


FIG. 2 Total PrP and proteinase-K-resistant PrP in *Prn-p^{0/0}* brains engrafted with PrP-producing neuroectodermal tissue. *a*, Brain histoblots. Top, immunodetection of PrP without previous proteinase K digestion. PrP is detectable in brain of both uninfected and infected grafted mice as well as in infected *tg20* but not in *Prn-p^{0/0}* mice. The rows below show histoblots digested with proteinase K at 20, 50 and 100 $\mu\text{g ml}^{-1}$ at 55 °C for 8 h. Grafted, inoculated *Prn-p^{0/0}* mice exhibit intense proteinase K-resistant PrP immunostaining within the infected *tg20* graft and patches of granular PrP immunoreactivity in the surrounding *Prn-p^{0/0}* host brain. Uninoculated grafts do not show immunoreactivity after proteinase K digestion. Bottom, schematics outlining graft (G), corpus callosum (CC), anterior commissure

(CA) and hippocampus (HI). In all cases, prior inoculation was ipsilateral to the graft. *b*, *c*, PrP immunoreactive deposits in grafted inoculated *Prn-p^{0/0}* mice were detected in the stratum radiatum and oriens of the ipsilateral and sometimes contralateral hippocampus (*b*), and occasionally also in cortical areas (*c*). *d*, a section of the same graft analysed in *c* shows diffuse immunoreactivity, plaque-like deposits (arrows) and intracellular deposits in vacuolated cell with neuronal morphology.

METHODS. Histoblots⁷ and immunohistochemistry⁸ were performed with PrP antisera 1B3 (ref. 22) or R340 (raised against recombinant *E. coli* PrP; Y. Kobayashi, unpublished results) and yielded identical results.

late stages (280–480 days p.i.), cellular density increased from 900 to over 4,000 cells mm^{-2} (Fig. 1*m–o*). Astrocytes constituted the main cell population and synaptophysin immunoreactivity was almost completely absent.

Although grafts had extensive contact with the recipient *Prn-p^{0/0}* brain, histopathology never extended into host tissue, even at the latest stages (Fig. 1*o*; asterisks). Surprisingly, histoblot analysis⁷ of non-inoculated, engrafted *Prn-p^{0/0}* brain revealed that PrP immunoreactivity extended to the white matter of the recipient brains (Fig. 2*a*, left column). In infected grafts, PrP^{Sc} was detected in both grafts and recipient brain, where it formed fine granules along white-matter tracts and even in the contralateral

hemisphere (Fig. 2*a*; second column from the left). Further, immunohistochemistry revealed PrP deposits in the host hippocampus (Fig. 2*b,c*) and occasionally in the parietal cortex of all animals harbouring PrP-expressing grafts. Up to 35 clusters of PrP deposits per section appeared late in infection, each consisting of 25–120 globules of 2–4 μm diameter closely associated with astrocytic processes; no deposits were observed in inoculated or mock-inoculated non-grafted *Prn-p^{0/0}* brains or in *Prn-p^{0/0}* brains engrafted with *Prn-p^{0/0}* tissue. Although no histopathological changes were detected in the surroundings of these deposits, PrP deposition within the graft was intimately associated with typical scrapie pathology (Fig. 2*d*).

TABLE 1 Determination of scrapie infectivity

Source of infectivity	Days after inoculation	Transmission*	Incubation time of recipient (days)
(a) Standard prion inoculum†			
RML, 10^{-1}	—	4/4	59, 60, 63, 68
RML, 10^{-3}	—	2/2	66, 67
RML, 10^{-5}	—	4/4	84, 85, 85, 96
RML, 10^{-7}	—	1/3	109, >217, >217
RML, 10^{-9}	—	0/4	>217, >217, >217, >217
RML, 10^{-11}	—	0/3	>217, >217, >217
(b) Mouse tissue‡			
Graft region	245	2/2	73, 73
Contralateral frontal cortex	336	0/2	>170, >170
Contralateral frontal cortex	350	0/2	>170, >170
Contralateral frontal cortex	428	0/2	>170, >170
Contralateral frontal cortex	454	0/2	>170, >170
Contralateral parietal cortex	285	2/2	101, 120
Cerebellum	285	0/3	>170, >170, >170
Spleen	285	0/1	>170

Infectivity was determined by injecting 30- μ l samples intracerebrally into *tga20* mice and determining time to terminal disease. *a*, End-point titration in *tga20* mice inoculated with the RML strain of mouse scrapie prions. The relationship between incubation time and titre was determined approximately by inoculating groups of four mice with logarithmic dilutions of a standard RML prion preparation⁴. End-point titration²⁰ gave a titre of $\sim 8 \log - \text{LD}_{50}$ units ml^{-1} of 10% homogenate, similar to the values obtained with CD-1 mice⁴. At the end point, the incubation time to terminal disease was about 110 days. From the data an approximate regression curve ($y = 1.3 - 0.11x$; $r = 0.96$) was established²¹. *b*, Infectivity of tissues from *Pm-p*^{0/0} mice engrafted with neuroectoderm from *tga20* embryos and inoculated with RML mouse prions. Accumulation of PrP^{Sc} and severe histopathological changes were found in all grafts. Brain samples were dissected *in situ* after removal of the calvaria, taking care to avoid contamination by the graft. Using the calibration curves from *a*, a titre of $\sim 5.7 \log - \text{ID}_{50}$ units ml^{-1} (where ID_{50} is half-maximal infective dose) of 10% homogenate was found for the graft region and a value of about $1.5 \log - \text{ID}_{50}$ units for the contralateral parietal cortex.

* (Number of mice contracting scrapie)/(number of mice inoculated and not succumbing to intercurrent events).

† A 10% brain homogenate from mice terminally sick with scrapie that had been inoculated with the RML strain of mouse scrapie prions.

‡ Assayed as 1% homogenate.

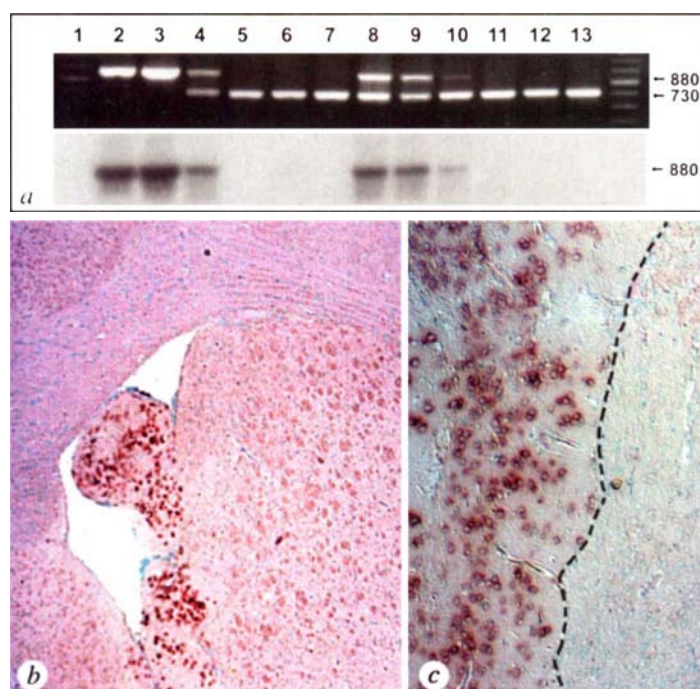
It is unlikely that such deposits were produced locally by *tga20* cells emigrating from the graft, because the graft borders were always sharply demarcated (Fig. 1). Analysis by polymerase chain reaction (PCR) of host brain regions containing PrP deposits failed to reveal PrP-encoding DNA (detection limit $< 1:10^4$; Fig. 3*a*) and graft-derived cells were never detected away from the graft by *in situ* hybridization (Fig. 3*b, c*) or by autoradiography of brain grafted with ³H-thymidine-labelled⁸ tissue (data not shown). We conclude that graft-derived PrP^C and PrP^{Sc} are transferred from the graft to distant areas of the host brain.

To determine the degree of infectivity, various portions of brains containing grafts with severe histopathology were inocu-

lated into *tga20* indicator mice. Samples of graft led to terminal scrapie after 74 days, indicating a titre of $\sim 5.7 \log - \text{LD}_{50}$ (half lethal dose) units per ml 10% homogenate (Table 1). Although frontal brain and cerebellum, in which no deposits were detected, showed no sign of infectivity, about $1.5 \log - \text{LD}_{50}$ units per ml 10% homogenate were detected in the contralateral hemisphere. Infectivity is not due to residual inoculum, because within 4 days of inoculation no infectivity can be detected in recipient brain⁴. Thus, infectious prions moved from the grafts to some regions of the PrP-deficient host brain without causing pathological changes or clinical disease. The distribution of PrP^{Sc} in the white-matter tracts of the host brain suggests diffusion within the extracellular

FIG. 3 No detectable migration of neuroectodermal cells grafted into host brain tissue. *a*, PCR analysis for PrP DNA in *Pm-p*^{0/0} tissue from different areas of a brain carrying a *tga20* graft. Top, ethidium-bromide-stained agarose gel. Bottom, Southern blot of the same gel. Lanes: 1 and 14, 100-bp ladder; 2, wild type; 3, *tga20* (on a *Pm-p*^{+/+} background); 4, *Pm-p*^{0/0} brain with *tga20* graft; 5, *Pm-p*^{0/0} brain without graft; 6 and 7, lateral and frontal portions of the *Pm-p*^{0/0} brain with *tga20* graft shown in lane 4; 8–13, logarithmic dilutions from $1:10^2$ to $1:10^7$ of wild-type brain in *Pm-p*^{0/0} brain DNA. At $1:10^4$ an 880-bp band is still visible (lane 10). *b, c*, *In situ* hybridization for *Pm-p*. The graft in *b* is intraventricular (20 $\times$ magnification) and in *c* is intraparenchymal (100 $\times$ magnification). Cells containing PrP mRNA are confined to the grafts.

METHODS. Brain DNA (10 ng) was used as a template for PCR amplification as described³. Southern blot analysis was performed using a randomly labelled 330-bp *Sma*I–*Av*II fragment corresponding to part of the *Pm-p* fragment deleted in *Pm-p*^{0/0} mice. The 730-bp band represents the disrupted *Pm-p* allele, the 880-bp band a non-disrupted gene. *In situ* hybridization was performed as published²³ using antisense RNA transcribed *in vitro* from the 330-bp *Sma*I–*Av*II fragment of the murine *Pm-p* gene.



space⁹ rather than axonal transport (S.B. and A.A., unpublished results).

It has been argued that scrapie pathology is due to intrinsic neurotoxicity of PrP^{Sc} rather than to depletion of PrP^C because *Pm-p^{0/0}* mice are normal in their development and behaviour^{3,10}, deposition of PrP-immunoreactive material colocalizes with typical histopathology¹¹, and synthetic amyloidogenic PrP fragments as well as liposome-packaged PrP²⁷⁻³⁰ (the protease-resistant core protein of PrP^{Sc}) are neurotoxic *in vitro*^{12,13}. However, even in regions adjoining the graft, which contained high levels of PrP^{Sc} and was clearly leaking such material, no scrapie pathology was observed in PrP^C-deficient tissue. Perhaps PrP^{Sc} is inherently non-toxic and PrP^{Sc} plaques found in spongiform encephalopathies are an epiphenomenon rather than a cause of neuronal damage. Indeed, the extent of PrP deposition in the brains of humans succumbing to prion diseases with similar clinical presentation is extremely variable^{14,15}. Alternatively, PrP^{Sc} is only toxic when it is formed and accumulated within the cell, but not when it is presented from outside. Finally, it may be that PrP^{Sc} is pathogenic when presented from outside, but only to cells expressing PrP^C, either because it initiates conversion of PrP^C to PrP^{Sc} at the cell surface and/or because it is internalized by way of association with PrP^C, which is endocytosed efficiently¹⁶. Considered together with previous transgenic studies^{17,18} that show delayed onset of disease in *Pm-p^{0/-}* mice despite massive accumulation of PrP^{Sc}, and with reports of typical scrapie histopathology in fatal familial insomnia-inoculated mouse brains in which PrP^{Sc} cannot be detected¹⁹, our

data imply that it is not deposition of PrP^{Sc} but rather the availability of PrP^C for some intracellular process elicited by the infectious agent that is directly linked to spongiosis, gliosis and neuronal death. □

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Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein

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FOLLOWING induction of experimental encephalomyelitis with a T-cell clone, L10C1, that is specific for the myelin basic protein epitope p87–99, the inflammatory infiltrate in the central nervous system contains a diverse collection of T cells with heterogeneous receptors. We show here that when clone L10C1 is tolerized *in vivo* with an analogue of p87–99, established paralysis is reversed, inflammatory infiltrates regress, and the heterogeneous T-cell infiltrate disappears from the brain, with

only the T-cell clones that incited disease remaining in the original lesions. We found that antibody raised against interleukin-4 reversed the tolerance induced by the altered peptide ligand. Treatment with this altered peptide ligand selectively silences pathogenic T cells and actively signals for the efflux of other T cells recruited to the site of disease as a result of the production of interleukin-4 and the reduction of tumour-necrosis factor- α in the lesion.

The T-cell clone L10C1 recognizes the myelin basic protein (MBP) epitope p87–99 and causes severe experimental encephalomyelitis (EAE) (Table 1). The third complementary-determining regions (CDR3) in the T-cell antigen receptor (TCR) α and β -chains of L10C1 are V β 6-PRGVGNQ and V α 1-SDTG, respectively. These sequences are homologous to VDJ β or VNJ α sequences in human or rat T-cell clones specific for MBP p87–99, and with VDJ β and VNJ α sequences found in multiple sclerosis (MS) lesions^{1–7}. We tolerized mice paralysed with EAE by targeting the T-cell clones that initiated disease; we then investigated the dynamics of the inflammatory infiltrates within the central nervous system (CNS).

(PL/JxSJL/J)F₁ mice received L10C1 and were treated with altered peptide ligand (APL) directly after transfer or after paralysis was established. Administration of the APL p87–99 (96P $\rightarrow$ A), in which a phenylalanine at residue 96 was replaced by alanine, prevented EAE or reversed paralysis (Table 1). This peptide binds poorly to I-A^b (Table 1) and induces a lower proliferative response in L10C1 than p87–99 (data not shown). In contrast, soluble non-stimulatory peptide analogues of p87–99, lacking residues critical for binding to the TCR or major histocompatibility complex (MHC), for example p87–99 (91K $\rightarrow$ A), p87–99 (92N $\rightarrow$ A), or p87–99 (93I $\rightarrow$ A), had no effect on disease (Table 1). The peptide p87–99 (90F $\rightarrow$ A) increased MHC binding relative to p87–99 and altered T-cell signalling (Table 1). A T-cell line reactive to p87–99 (90F $\rightarrow$ A) abrogated EAE induced with spinal cord homogenate (data not shown).

The mechanism causing tolerance was analysed. p87–99 (96P $\rightarrow$ A) is a partial agonist for L10C1 (Table 1). The therapeutic APL had no influence on the *in vitro* proliferation of L10C1 by p87–99 (data not shown), so MHC competition⁸ or TCR antagonism⁹ are unlikely to explain its action.

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TABLE 1 Stimulation of MBP p87–99-specific T-cell clone L10C1 by peptides with single amino-acid substitutions

Peptide	Substitution	Relative affinity for I-A ^b	Proliferation of L10C1	Treatment of EAE
87–99	VHFFKNIVTPRTP	++	+++	–
87–99	87 A AHFFKNIVTPRTP	++	++	ND
87–99	88 A VAFFKNIVTPRTP	++	–	ND
87–99	89 A VHAFKNIVTPRTP	+	–	ND
88–99	90 A VHFAKNIVTPRTP	+++	–	–
87–99	91 A VHFFANIVTPRTP	ND	–	–
87–99	92 A VHFFKAIVTPRTP	–	–	–
87–99	93 A VHFFKNAVTPRTP	–	–	–
87–99	94 A VHFFKNIAVTPRTP	–	–	ND
87–99	95 A VHFFKNIVAPRTP	+++	+	ND
87–99	96 A VHFFKNIVTARTP	+	+	–
87–99	97 A VHFFKNIVTPATP	ND	+	ND
87–99	98 A VHFFKNIVTPRAP	ND	+	ND
87–99	99 A VHFFKNIVTPRTA	ND	–	ND

Human MBP peptide p87–99 and a series of single alanine-substituted analogues were synthesized as described¹⁹. The relative affinity of peptides was determined using the I-A^b-expressing B-cell lymphoma line LS102.9. The line was maintained in IMDM medium (Gibco) supplemented with 5% FCS (Flow Lab), 2 mM L-glutamine (Gibco), 5×10^{-5} M 2-mercaptoethanol, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (IMDM-5). Cells were collected while subconfluent and distributed at 2×10^5 cells per well of a 96-well microtitre plate. Cells were resuspended in IMDM-5 containing a top concentration of the biotinylated peptide, Bio87–99, of 100 µM. Alanine-substituted analogues of p87–99 were added to 300 µM and cells were incubated at 37 °C for 75 min. Unbiotinylated p87–99 was used as a positive control for competition binding studies; the I-A^b-restricted peptide Ac1-9 (4K→Y) was used as a negative control. LS102.9 cells were then washed three times in PBS supplemented with 1% FCS and 2 mM Na₂S₂O₃ (washing solution) before being incubated on ice in ExtrAvidin–FITC (Sigma) for 30 min. Cells were washed thoroughly before being fixed in 1% formaldehyde (BDH). Three thousand events from each sample were analysed on a Becton Dickinson FACSort using Lysis II software and mean channel fluorescence was used as a measure of fluorescence intensity. Binding was scored as follows: –, no binding; –, binding with an affinity intermediate between Ac1-9 (4K→Y) and p87–99; ++, binding equivalent to p87–99; + + +, binding with an affinity greater than p87–99. T-cell proliferation was assayed as described²⁰ and the transfer of EAE by T-cell clone L10C1 and treatment with soluble peptide analogues are described in Fig. 1 legend. T-cell responses were graded as: +, proliferation at 40 µM peptide; + –, proliferation at 4 µM peptide; + + +, proliferation at 1 µM peptide. Treatment of EAE: + refers to the ability to prevent or reverse ongoing EAE (see legends to Figs 1 and 2) by injection of the APL or native p87–99 intraperitoneally. ND, not determined.

In EAE, inflammatory infiltrates are heterogeneous¹⁰, with a diverse array of TCR V-region genes being seen seven days after the transfer of L10C1 (Table 2a). When treated with APL after onset of paralysis, the diversity of the TCR transcripts in infiltrates was reduced in the spinal cord, whereas the different TCR transcripts disappeared within 48 h in the brain (Table 2a). Furthermore, tumour-necrosis factor (TNF)-α transcripts, which are associated with pathogenicity of T-cell clones^{11,12}, cannot be detected in brain after treatment with APL (Table 2b). When scoring inflammatory infiltrates, there were significant differences between mice treated with p87–99 (96P → A) compared to controls (data not shown).

Direct sequencing of Vβ6 transcripts from brains of diseased

animals after reversal of EAE demonstrated that the TCR VDJβ sequence, Vβ6PRGVGNQ, of residual cells in the lesions was identical to that of the clone initially transferred into the mice. Thus, the disease mediated by the T-cell clone can still be positively identified *in situ* after treatment with APL, whereas other inflammatory T cells had disappeared. Because it was possible directly to sequence Vβ6-Cβ PCR products from the EAE lesion, there must have been little or no diversity in Vβ6 TCR transcripts following APL treatment. The presence of the encephalitogenic clone in the lesion of animals successfully treated with the APL is insufficient to maintain clinical disease. These residual T cells have been silenced. The presence of full-length Vβ6-D-J-Cβ sequences indicates that apoptosis has not

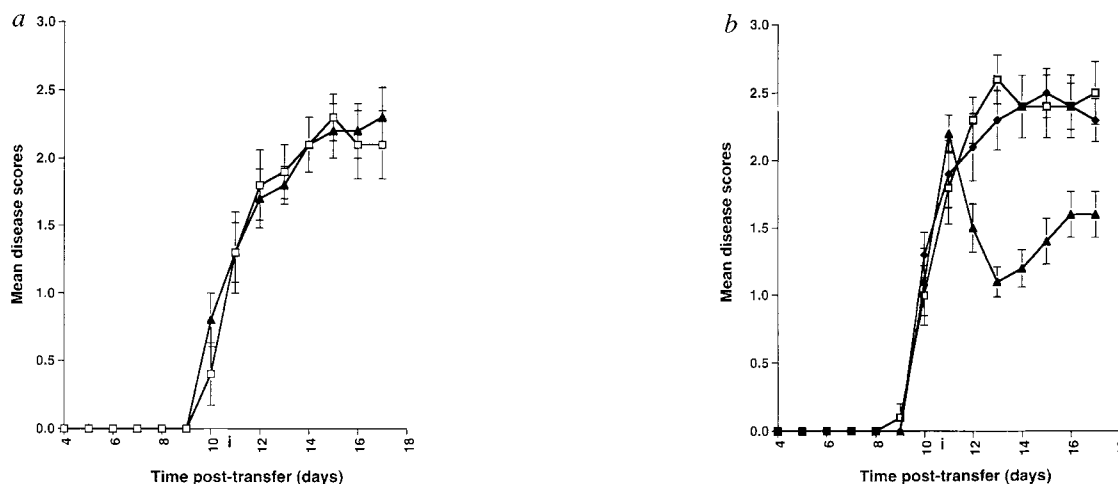


FIG. 1 Reversal of adoptively transferred EAE with MBP analogue p87–99(96P → A). *a*, Soluble MBP analogue p87–99 (96P → A) has no effect on EAE mediated by MBP Ac1-11-specific T-cell line L9. Ten animals per group were treated with PBS alone (squares) or with 0.5 mg soluble p87–99(96P → A) (triangles) in 0.5 ml PBS. *b*, Soluble MBP analogue p87–99(96P → A) reduces severity of EAE mediated by the Ac1-11-specific T-cell line L9 when T-cell clone L10C1 is transferred as well. Ten animals per group were treated with PBS alone (squares), with 0.5 mg soluble p87–99(91K → A) (diamonds) in 0.5 ml PBS, or 0.5 mg soluble p87–99(96P → A) (triangles) in 0.5 ml PBS (i, time of i.p. injection of peptides). METHODS. EAE was induced by transferring 3×10^6 T cells of the MBP Ac1-

11 specific T-cell line L9 (ref. 20). On day 6 after transfer, groups of mice shown in *b* were injected with 5×10^6 T cells of T-cell clone L10C1. Eleven days after the first transfer to induce EAE, mice were injected i.p. with 0.5 mg in 0.5 ml PBS of MBP peptide analogues p87–99(96P → A), p87–99(91K → A) or PBS alone, as indicated. Mice were scored daily according to the following scale: 0, no clinical disease; 1, tail weakness; 2, mono- or paraparesis (incomplete paralysis of one or two hindlimbs); 3, mono- or paraplegia (complete paralysis of one or two hindlimbs); 4, paraplegia with forelimb weakness or paralysis; 5, moribund or dead animals. Data are given as mean disease scores $\pm$ s.e.

TABLE 2 TCR V β and TNF- α gene expression in the CNS of (PL/JxSJL/J) F₁ mice after treatment with p87-99 peptide analogues

Treatment/sample	1	2	3	4	5	6	7	8	9	10	TCR V β 11	12	13	14	15	16	17	18	19
p87-99 (96P→A)																			
Spinal cord 1	+	+	+			+		+		+		+	+			+	+		
Spinal cord 2		+				+			+				+						
Control peptides																			
Spinal cord 3	+	+				+		+	+			+	+			+			
Spinal cord 4	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+		+	
P87-99 (96p→A)																			
Brain 1						+					+								
Brain 2						+													
Control peptides																			
Brain 3	+	+				+	+	+	+							+		+	
Brain 4	+					+		+			+	+		+	+		+		
Treatment/sample	TNF- α					β -Actin													
p87-99 (96P→A)																			
Spinal cord 1				+				+											
Spinal cord 2				+				+											
Control peptides																			
Spinal cord 3				+				+											
Spinal cord 4				+				+											
p87-99 (96P→A)																			
Brain 1				-				+											
Brain 2				-				+											
Control peptides																			
Brain 3				+				+											
Brain 4				+				+											

The encephalitogenic T-cell clone L10C1 was activated *in vitro* with MBP p87-99 and antigen-presenting cells and adoptively transferred into mice. All mice developed paralysis on day 7 after transfer, with an EAE score of at least 2. On day 7 and/or 8 and 9, mice were treated intraperitoneally with 0.5 mg of soluble peptide analogues p87-99(96P→A) or soluble control peptides (p87-99(92N→A) or p87-99(93I→A)) as indicated. 48 h after treatment, mice were killed by CO₂ inhalation and then perfused with 30 ml PBS and their brains and spinal cords dissected. Reverse-transcribed mRNA isolated from CNS tissue was analysed by PCR using either TCR V β primers specific for 19 different mouse TCR V β regions and a single common C β primer, or β -actin primers and mouse TNF- α -specific primers. Perfusion of animals, preparation and storage of CNS tissue, RNA and cDNA preparation from tissue samples, PCR amplification of cDNA using β -actin primers and TCR V β primers specific for 19 different mouse TCR V β regions have all been described²⁰. Primers were synthesized by Operon Technologies (Alameda, CA). TCR products amplified by PCR were confirmed by Southern blotting using a C β -specific probe. PCR amplification of CNS tissue derived RNA/cDNA using mouse TNF- α -specific primers (TNF- α sense primer: 5'-GGC AGG TCT ACT TTG GAG TCA TTG C-3'; TNF- α antisense primer: 5'-ACA TTC GAG GCT CCA GTG AAT TCG G-3) has been described²¹.

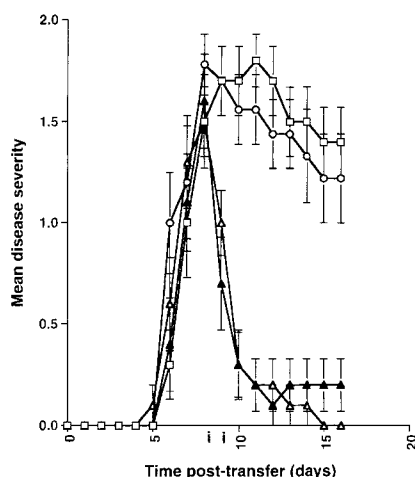


FIG. 2 Reversal of adoptively transferred EAE with MBP analogue p87-99(96P→A) is dependent on IL-4 *in vivo*. Nine to ten animals per group were injected with 10×10^6 T cells of T-cell clone L10C1. On day 8 and 9 after transfer, mice were treated with 0.5 ml PBS (squares) or 0.5 mg soluble p87-99(96P→A) (triangles and circles) in 0.5 ml PBS. Two hours later, PBS (black triangles), 1 mg HPLC-purified mAbs 11B11 (rat anti-mouse-IL 4, IgG1, circles) or GL113 (rat-anti-mouse- β -galactosidase, IgG1; open triangles) were injected i.p. as indicated in (i, time of i.p. injection of peptides and antibodies). Mice were scored daily as described in Fig. 1 legend. Data are given as mean disease scores $\pm$ s.e.

deleted all T-cell clones responsible for inciting disease. We searched for other mechanisms underlying tolerance.

We asked whether antigen-specific immunotherapy with APL therapy is confined to the specific T cells that recognize APL. If so, the therapeutic potential of this approach would be limited in the case of diverse antigen reactivity¹³. Figure 1 shows that EAE mediated by MBPpAc1-11-specific T cells can be ameliorated by targeting p87-99-specific T cells with p87-99 (96P→A). This protection only occurs if L10C1 T cells, reactive to p87-99, are present in the lesions after co-transfer (Fig. 1b), indicating that APL can directly influence T cells with other specificities in an established lesion. The *trans*-acting effect depends on the intralesional presence of a T-cell clone that recognizes the APL.

Reversal of EAE with p87-99 (96P→A) depends on the availability of the cytokine interleukin-(IL)-4. Neutralizing monoclonal antibodies directed against IL-4 block the therapeutic effect of APL (Fig. 2). In parallel studies, EAE induced with proteolipid protein can be reversed at the onset of paralysis with the proteolipid protein PLP p139-151 given intraperitoneally. This effect is abrogated when anti-IL-4 is given together with p139-151 (data not shown).

L10C1 produced both IL-4 and TNF- α . T cells (10^6 per ml medium) from L10C1 were cultured for 36 hours, together with 10×10^6 irradiated syngeneic splenocytes and 10 μ M MBP p87-99 or p87-99 (P→A): $18,680 \pm 57$ pg ml⁻¹ or 880 ± 57 pg ml⁻¹, respectively, of TNF- α and 77.5 ± 3.5 pg ml⁻¹ or 13.5 ± 2.1 pg ml⁻¹, respectively, of IL-4 were detected. Thus the APL p87-99 (96P→A) shifts the ratio between TNF- α and IL-4 by $\sim$ 4-fold. IL-4 is a potent inhibitor of TNF- α (ref. 14).

Inflammation is a dynamic process, and T-cell tolerance with APL leads to the regression and dissolution of an inflammatory

infiltrate. This phenomenon is mediated by the downregulation of the production of TNF- α and by the action of IL-4. TNF- α is a mediator of cell recruitment in inflammatory infiltrates¹⁵ and is a critical cytokine in the pathogenesis of EAE, and probably of multiple sclerosis¹⁶; IL-4 is associated with recovery from disease, and is seen in multiple sclerotic lesions^{17,18}. By selectively targeting the initial trigger for inflammation, the secondary infiltrate can be controlled. This may be relevant for highly selective immune therapy of autoimmune disease in the face of determinant spreading¹³ and nonspecific amplification of the inflammatory response. □

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Augmented humoral and anaphylactic responses in Fc γ RII-deficient mice

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DESPITE its widespread distribution on both lymphoid and myeloid cells, the biological role of the low-affinity immunoglobulin-G receptor, Fc γ RII, is not fully understood. Defects in this receptor or its signalling pathway in B cells result in perturbations in immune-complex-mediated feedback inhibition of antibody production^{1–6}. We now report that Fc γ RII-deficient animals display elevated immunoglobulin levels in response to both thymus-dependent and thymus-independent antigens. Additionally, the effector arm of the allergic response is perturbed in these mice. Mast cells from Fc γ RII^{−/−} are highly sensitive to IgG-triggered degranulation, in contrast to their wild-type counterparts. Fc γ RII-deficient mice demonstrate an enhanced passive cutaneous anaphylaxis reaction, the result of a decreased threshold for mast-cell activation by Fc γ RIII cross-linking. These results demonstrate that Fc γ RII acts as a general negative regulator of immune-complex-triggered activation *in vivo* for both the afferent and efferent limbs of the immune response. Exploiting this property offers new therapeutic opportunities for the treatment of allergic and autoimmune disorders.

Immunoglobulin Fc receptors (FcRs) constitute a family of haematopoietic cell-surface molecules capable of eliciting intracellular signals and triggering numerous effector responses upon crosslinking by their ligand, the antibody–antigen complex⁷. Types I and III FcRs for IgG (Fc γ RI, Fc γ RIII) are expressed primarily on cells of the myeloid lineage and mediate effector functions, including phagocytosis, antibody-dependent cellular cytotoxicity and the release of inflammatory mediators, whereas type II receptors (Fc γ RII) are expressed on both myeloid and lymphoid lineages. *In vitro*, Fc γ RII inhibits the activation signal generated by the B-cell antigen receptor^{1–4}, mediated in part through the recruitment and activation of protein tyrosine phosphatases^{5,6}. Similarly, reconstitution studies have suggested that Fc γ RII can inhibit activation through other, immune receptor tyrosine activation motif (ITAM)-containing receptors⁸. We therefore investigated the *in vivo* consequences of disrupting this inhibitory pathway.

Mice homozygous for Fc γ RII^{−/−} were generated (Fig. 1a,b), which developed normally and were fertile. The absence of messenger RNA (not shown) and protein (Fig. 1c) for Fc γ RIIB was demonstrated by northern and western blotting, respectively; the absence of membrane bound Fc γ RIIB was demonstrated by flow cytometry of splenic B cells (Fig. 1d).

Expression of Fc γ RIIB on effector cells has been shown to depend on their state of activation^{9,10}. Resident peritoneal macrophages (Fig. 1e) and bone-marrow-derived mast cells (Fig. 1f) reveal a >90% reduction in surface staining with the monoclonal antibody 2.4G2 in Fc γ RII^{−/−} mice (Fig. 1e,f), whereas thioglycollate-elicited macrophages retain 80% of their 2.4G2 staining (data not shown).

Development of myeloid and lymphoid cell lineages were not affected by the loss of Fc γ RII, as revealed by flow cytometric analysis of cells derived from the spleen, bone marrow, thymus and peritoneal cavity using a panel of lineage-specific markers (data not shown).

Crosslinking of surface IgM on resting B cells *in vitro* induces a proliferative response only when anti- μ F(ab')₂ is used as the stimulating antibody; intact antibody fails to stimulate B cell receptor-mediated cellular activation¹. In contrast, B cells derived from Fc γ RII^{−/−} proliferate in response to both intact anti- μ and F(ab')₂ (Fig. 2a). The significance of this pathway *in vivo* was determined by immunizing wild-type and Fc γ RII^{−/−} animals with sheep red blood cell (SRBC) or trinitrophenol keyhole limpet haemocyanin (TNP-KLH) as thymus-dependent antigens (Fig. 3a,b) or trinitrophenol lipopolysaccharide (THP-LPS) or TNP-Ficoll as thymus-independent antigens (Fig. 3c,d). In all cases, Fc γ RII^{−/−} showed significantly higher antibody titres than those of wild-type littermates (Fig. 3). This enhancement was evident after 14 days and persisted during the secondary response. Anti-SRBC IgM and IgG plaque-forming cell responses of splenocytes derived from deficient mice were significantly higher than those of wild-type mice at four days after the second immunization (data not shown). Isotype-specific enzyme-linked immunosorbent assays (ELISAs) indicated that the immunoglobulin isotypes that contribute to these differences were IgM, IgG1, 2a, 2b, 3 and IgA (data not shown).

Recent *in vitro* reconstitution studies in mast cell lines has suggested that Fc γ RII could inhibit FcR-triggered activation⁸. Bone-marrow mast cells derived from Fc γ RII-deficient or wild-type mice were stimulated through Fc ϵ RI or Fc γ RIII (Fig. 2b). Degranulation and serotonin release in response to IgE cross-linking were similar in wild-type and Fc γ RII-deficient mice. In contrast, wild-type mast cells show little degranulation or serotonin release above background upon crosslinking of Fc γ RII/III, whereas mast cells from Fc γ RII-deficient mice showed a substantial activation upon the same stimulation (Fig. 2b). Hence, Fc γ RII is sufficient substantially to repress mast-cell activation triggered by Fc γ RIII, but has no direct effect on Fc ϵ RI-mediated activation. Loss of Fc γ RII thus renders these cells sensitive to IgG-mediated triggering. IgG1-mediated passive cutaneous anaphylaxis (PCA)

in wild-type and $\text{Fc}\gamma\text{RII}$ deficient mice was evaluated (Fig. 4) to determine the potential role of $\text{Fc}\gamma\text{RII}$ in modulating this response *in vivo* (Fig. 4). PCA reactions were performed with a series of IgG concentrations, containing amounts ranging from 4 to 0.125 μg . Significant augmentation of this mast-cell-mediated response at a 5–10-fold lower antibody concentration was observed in $\text{Fc}\gamma\text{RII}$ -deficient mice as compared to wild-type littermate controls.

Our results indicate that $\text{Fc}\gamma\text{RII}$ functions as an inhibitory receptor for both B cells and mast cells *in vivo*. Inactivation of this receptor results in a defect in the ability to regulate antibody levels in response to antigenic stimulation for IgM, IgG and IgA, implying a network of interactions which are dependent on IgG immune complexes. Defects in $\text{Fc}\gamma\text{RII}$ function may therefore contribute to the development of autoimmunity by a failure of

feedback inhibition to regulate antibody production, as suggested by the analysis of the 'motheaten' (*me*) mouse, which is defective in protein tyrosine phosphatase 1C (refs. 5, 6, 11, 12). Loss of $\text{Fc}\gamma\text{RII}$ does not, however, result in uncontrolled antibody production after antigenic challenge *in vivo*, implying that additional pathways for maintaining antibody homeostasis are operating. The inhibitory role for $\text{Fc}\gamma\text{RII}$ on mast cells suggests that mast-cell activation by IgE may also be inhibited by recruitment of $\text{Fc}\gamma\text{RII}$, as might occur by IgG blocking antibodies in allergic desensitization. Indeed, co-ligation of $\text{Fc}\epsilon\text{RI}$ to $\text{Fc}\gamma\text{RII}$ renders mast cells anergic (M.O. and J.V.R., unpublished observations) and suggests new therapeutic methods by which mast cells may be repressed. $\text{Fc}\gamma\text{RII}$ -deficient mice are therefore an important tool for the dissection of both afferent and efferent arms of the immune response. □

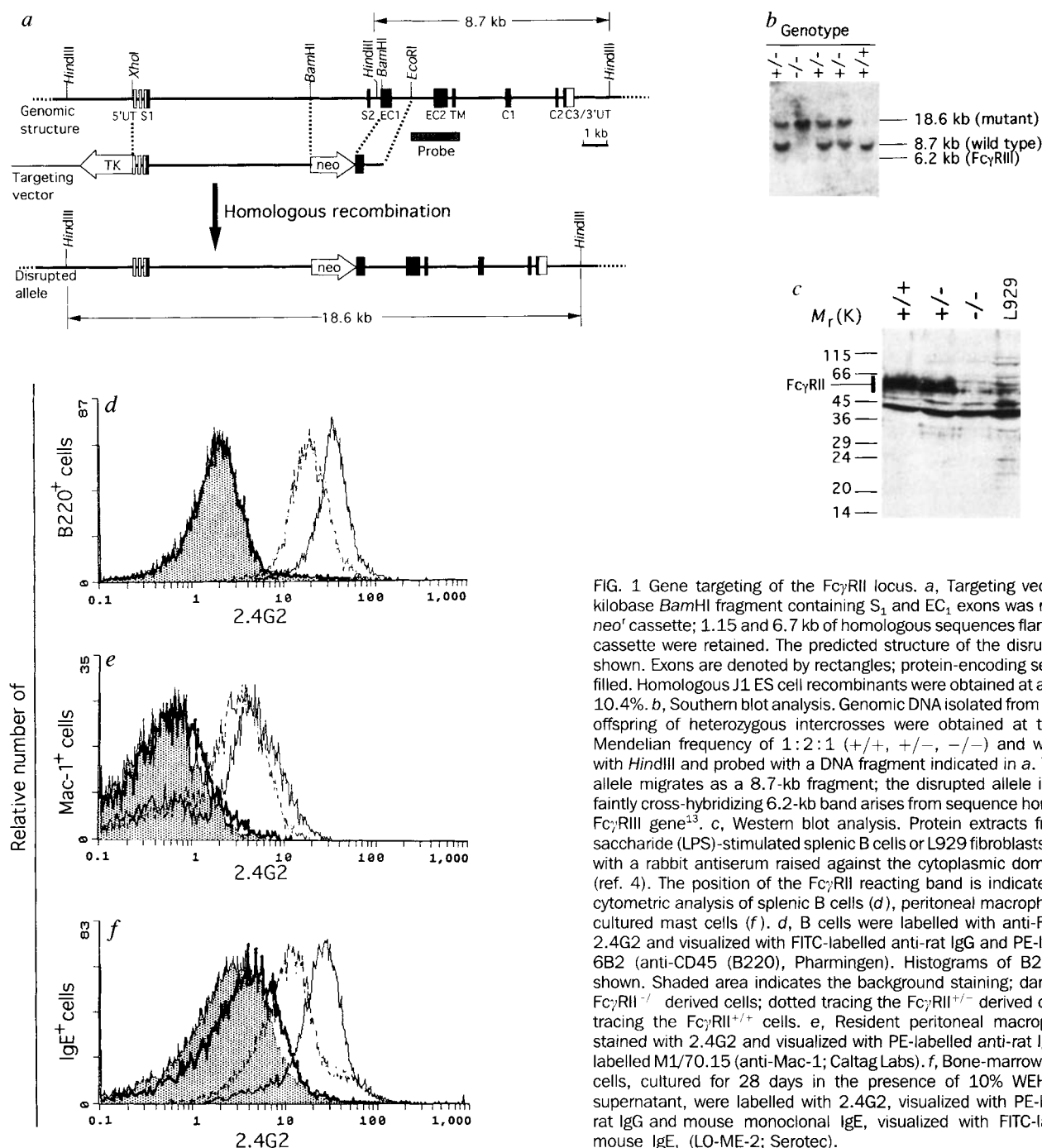


FIG. 1 Gene targeting of the $\text{Fc}\gamma\text{RII}$ locus. **a**, Targeting vector. A 2.65-kilobase *Bam*HI fragment containing S_1 and EC_1 exons was replaced by a *neo* cassette; 1.15 and 6.7 kb of homologous sequences flanking the *neo* cassette were retained. The predicted structure of the disrupted allele is shown. Exons are denoted by rectangles; protein-encoding sequences are filled. Homologous J1 ES cell recombinants were obtained at a frequency of 10.4%. **b**, Southern blot analysis. Genomic DNA isolated from the tail tips of offspring of heterozygous intercrosses were obtained at the predicted Mendelian frequency of 1:2:1 (+/+, +/-, -/-) and were digested with *Hind*III and probed with a DNA fragment indicated in **a**. The wild-type allele migrates as a 8.7-kb fragment; the disrupted allele is 18.6-kb. A faintly cross-hybridizing 6.2-kb band arises from sequence homology to the $\text{Fc}\gamma\text{RIII}$ gene¹³. **c**, Western blot analysis. Protein extracts from lipopolysaccharide (LPS)-stimulated splenic B cells or L929 fibroblasts were probed with a rabbit antiserum raised against the cytoplasmic domain of $\text{Fc}\gamma\text{RII}$ (ref. 4). The position of the $\text{Fc}\gamma\text{RII}$ reacting band is indicated. **d–f**, Flow cytometric analysis of splenic B cells (**d**), peritoneal macrophages (**e**) and cultured mast cells (**f**). **d**, B cells were labelled with anti- $\text{Fc}\gamma\text{RII/III}$ mAb 2.4G2 and visualized with FITC-labelled anti-rat IgG and PE-labelled RA3-6B2 (anti-CD45 (B220), Pharmingen). Histograms of B220 cells are shown. Shaded area indicates the background staining; dark tracing the $\text{Fc}\gamma\text{RII}^{-/-}$ derived cells; dotted tracing the $\text{Fc}\gamma\text{RII}^{+/+}$ derived cells and thin tracing the $\text{Fc}\gamma\text{RII}^{+/+}$ cells. **e**, Resident peritoneal macrophages were stained with 2.4G2 and visualized with PE-labelled anti-rat IgG and FITC-labelled M1/70.15 (anti-Mac-1; Caltag Labs). **f**, Bone-marrow derived mast cells, cultured for 28 days in the presence of 10% WEHI-3A culture supernatant, were labelled with 2.4G2, visualized with PE-labelled anti-rat IgG and mouse monoclonal IgE, visualized with FITC-labelled anti-mouse IgE, (LO-ME-2; Serotec).

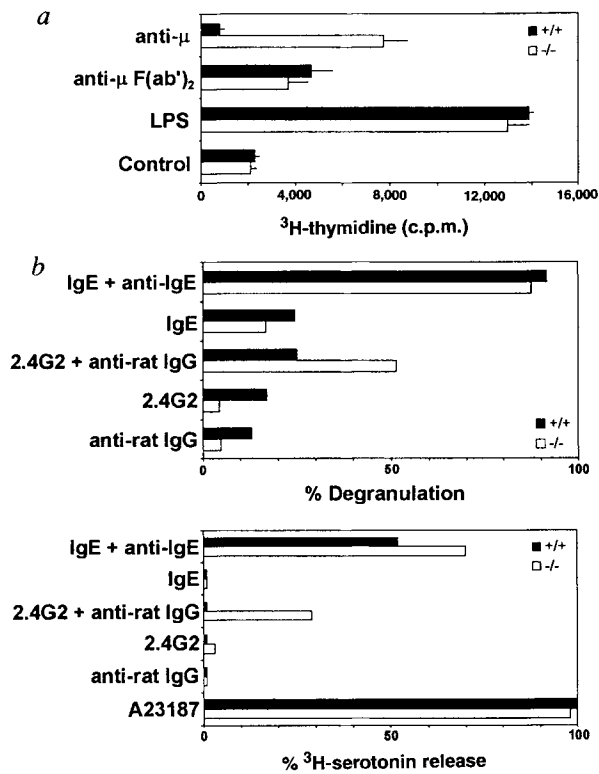


FIG. 2 B-cell and mast-cell stimulation *in vitro*. *a*, B-cell proliferative response to anti-μ stimulation. Splenic B cells were depleted of T cells with anti-Thy 1.2, anti-CD4, anti-CD8 and anti-CD-3 plus rabbit complement. The enriched B cells were stimulated with 5 μg ml⁻¹ LPS, 20 μg ml⁻¹ rabbit anti-mouse IgM (μ-specific; Serotec) or 20 μg ml⁻¹ goat anti-mouse IgM F(ab')₂. Mean [³H]thymidine uptake at 30–48 h and s.d. of triplicated samples for wild-type (+/+) and FcγRII-deficient (-/-) are shown. *b*, Mast-cell stimulation. Cultured mast cells were stimulated with 10 μg ml⁻¹ IgE plus 10 μg ml⁻¹ anti-mouse IgE, 5 μg ml⁻¹ 2.4G2 plus 20 μg ml⁻¹ anti-rat IgG (Cappel) or 5 μg ml⁻¹ ionophore (A23187). Per cent degranulation and [³H]serotonin release were determined 1 h after stimulation as described^{14,15}.

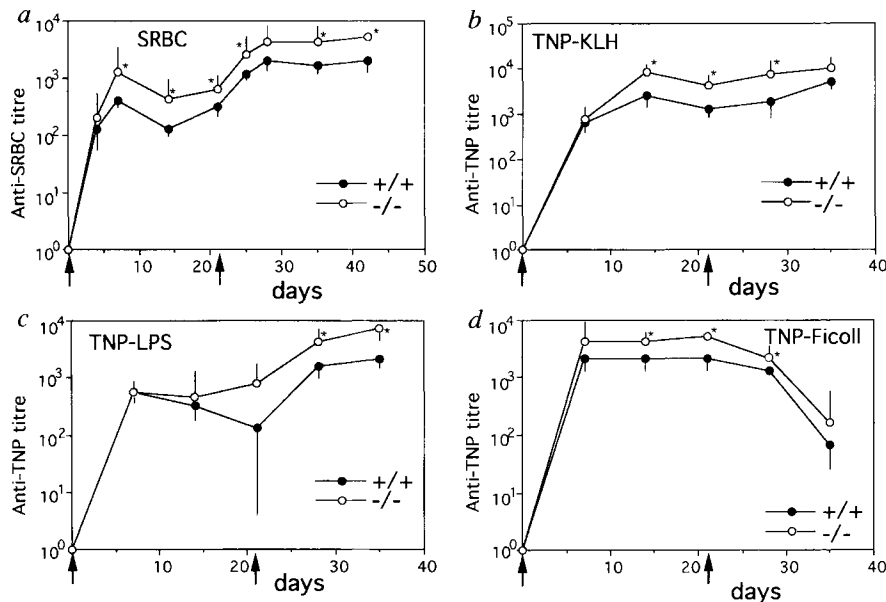


FIG. 3 Humoral response to antigen challenge in FcγRII-deficient mice. Wild-type (+/+) and FcγRII-deficient (-/-) mice were injected intraperitoneally with 2 × 10⁸ SRBC (*a*), 100 μg TNP KLH in complete Freund's adjuvant (*b*), 50 μg TNP-LPS (*c*) or 25 μg TNP-Ficoll (*d*) at day 0 and bled at the indicated times. Serum antibody levels were determined as haemagglutination titres using SRBC (*a*) or TNP-SRBC (*b-d*). Animals were boosted at 21 days to elicit a secondary response (denoted by arrow). The asterisk denotes values significant at *P* < 0.05. Experiments in *a* were repeated 5 times with similar results. Experiments in *b-d* were done twice.

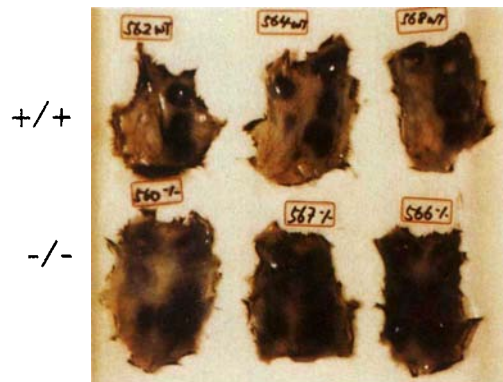


FIG. 4 IgG-mediated passive cutaneous anaphylaxis is greatly enhanced in FcγRII-deficient mice. Wild-type (+/+) and FcγRII-deficient (-/-) mice (in triplicate) were injected intradermally with mouse anti-TNP IgG1 ranging from 4 μg to 0.125 μg per site in steps of 2-fold dilution. The injections were arranged to contain decreasing amounts starting at the upper right (4 μg) to the lower left (0.125 μg) for each animal. One hour later mice were injected intravenously with 100 μg TNP-ovalbumin with 1% Evans blue. Animals were killed 30 min after intravenous challenge and the injected skin analysed for extravasation of blue dye, an indication of vascular leakage¹⁶.

Note added in proof: Since submission of this study, data have been presented¹⁷ which further substantiates the role of FcγRII as a genome inhibitor of ITAM-triggered activation.

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Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes

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DYSREGULATION of apoptosis can result in inappropriate suppression of cell death, as occurs in the development of some cancers¹, or in failure to control the extent of cell death, as is believed to occur in acquired immunodeficiency and certain neurodegenerative disorders, such as spinal muscular atrophy (SMA). Recently, we isolated a candidate gene, encoding neuronal apoptosis inhibitor protein (NAIP)², for SMA. This gene is homologous to two baculovirus inhibitor of apoptosis proteins^{3,4} (Cp-IAP and Op-IAP) and is partly deleted in individuals with type I SMA. A second SMA candidate gene encoding survival motor neuron (SMN), which is contiguous with the NAIP locus on 5q13.1, was also reported⁵. Here we demonstrate a NAIP-mediated inhibition of apoptosis induced by a variety of signals, and have identified three additional human complementary DNAs and a *Drosophila melanogaster* sequence that are also homologous to the baculovirus IAPs. The four open reading frames (ORFs) possess three baculoviral inhibition of apoptosis protein repeat (BIR) domains and a carboxy-terminal RING zinc-finger. The human *iap* genes have a distinct but overlapping pattern of expression in fetal and adult tissues. These proteins significantly increase the number of known apoptotic suppressors.

To analyse the effect of NAIP on apoptosis, expression plasmids, either alone or encoding full-length NAIP or Bcl-2, were transfected into CHO, Rat-1 and HeLa cells followed by G418 selection. In a second approach, cells were infected with adenovirus, alone or expressing either NAIP, antisense NAIP or LacZ. Expression of NAIP was confirmed in both procedures by western blot analysis and immunofluorescence (data not shown). After infection with the recombinant adenoviruses, CHO cells were induced to undergo apoptosis by serum deprivation with survival rates of 48% (no insert), 51% (LacZ) and 45% (antisense NAIP) at 48 h (Fig. 1a). In contrast, CHO cells infected with adenovirus expressing NAIP demonstrate 78–83% survival. NAIP also induced survival in stably transfected CHO pools, albeit slightly less than is seen in adenovirus-infected cells: 44% of the vector transfectants and 65% of the NAIP transfectants survived at 48 h (Fig. 1b).

Overexpression of NAIP in CHO cells treated with 20 μM menadione (a potent inducer of free radicals⁶) resulted in 20–30% enhancement of survival compared with controls after 24 h (Fig. 1c, d). Overexpression of NAIP also protected menadione-treated Rat-1 fibroblasts from undergoing cell death (Fig. 1e–h). Only 15% of cells infected with LacZ expressing adenovirus were viable at 12 h, in contrast to 80% of NAIP-infected cells, an effect also detected with the pooled Rat-1 NAIP transfectants. Even greater survival was induced by NAIP overexpression at a lower menadione concentration (5 μM), with 98% of pooled NAIP transfectants and 33% of control transfectants viable at 24 h (Fig. 1g, h).

We next assessed the protective effect of NAIP on cells exposed to the cytokine tumour necrosis factor (TNF)-α. HeLa cells treated with TNF-α and cycloheximide were protected from apoptosis when infected with adenovirus expressing high levels of NAIP (139% survival at 48 h) at 48 h, an effect not observed with antisense NAIP (52% survival) (Fig. 1i, j). A similar effect was observed in pooled HeLa transformants. To confirm that cells surviving the apoptotic agents expressed NAIP, immunofluorescence with anti-NAIP antisera was performed on several of the cell death assays. A dramatic enrichment of NAIP-expressing cells was observed, with no alteration noted in the cytoplasmic distribution of NAIP (data not shown). Having demonstrated the apoptotic suppression activity of NAIP, we next sought to identify other human genes that might encode IAP-like proteins.

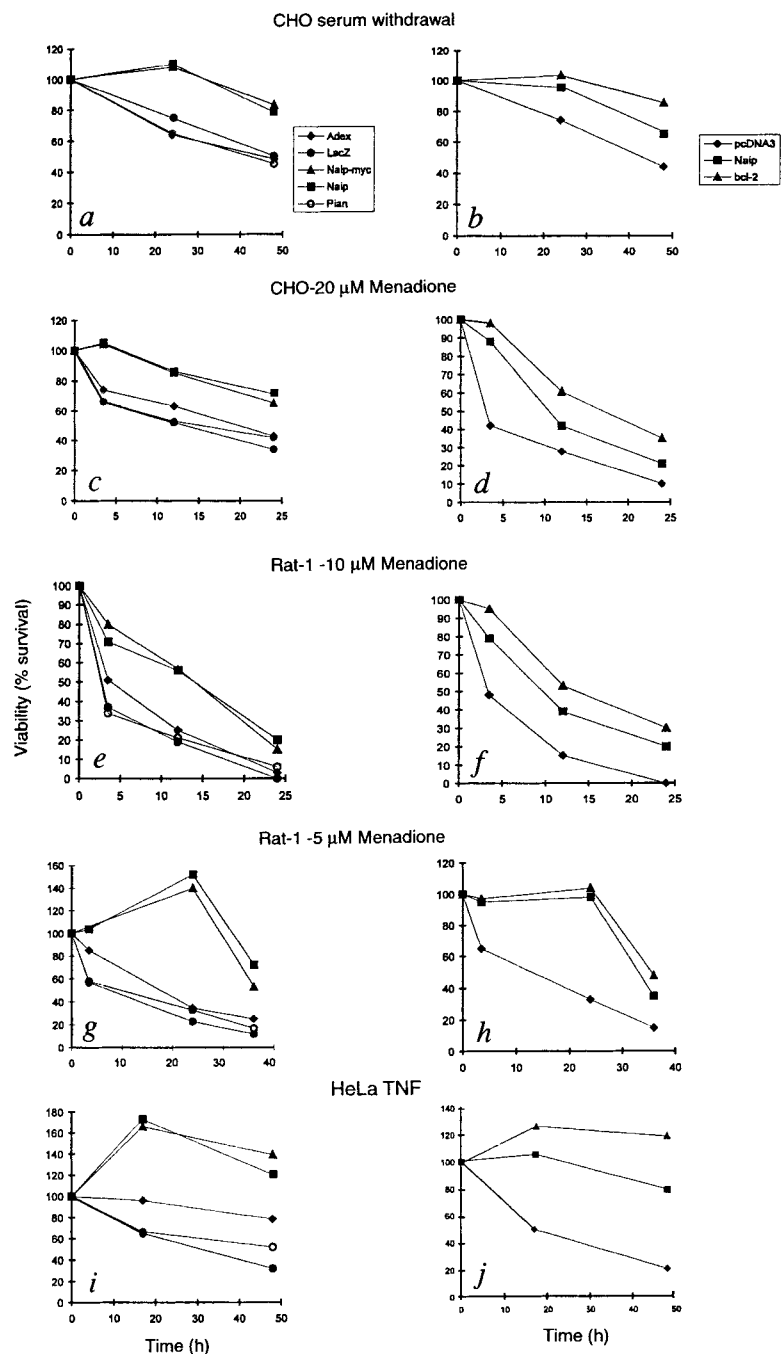
An initial search of the protein databases at NIH using the Blast program⁷ with IAP sequences from *Cydia pomonella* identified a homologous sequence-tagged site of 273 bp that encodes a potential RING zinc-finger domain located on the X chromosome at Xq24-25. A 240-bp product of polymerase chain reaction (PCR), generated using primers within this sequence, was used to probe a λZap-II human fetal brain cDNA library (Stratagene). Clones were isolated and sequenced, and the resulting cDNA, which encodes a protein with three BIR motifs and a RING zinc-finger, was termed *xiap* (for X-linked IAP). A search of the databases with *xiap* sequences led to the identification of an additional GenBank submission (accession number T96284), containing a 410-bp sequence with moderate homology to the *xiap* BIR domain sequence. A 159-bp fragment amplified by PCR from this sequence was used to screen a λUniZap human liver cDNA library (Stratagene), resulting in the isolation of two new human *iap* cDNAs, *hiap-1* and *hiap-2*. Both contain three BIR motifs and a RING zinc-finger (Fig. 2). In addition to the human IAP proteins, a *Drosophila* genomic DNA sequence (GenBank accession number M96581) was identified with the potential to encode a previously unrecognized IAP protein. By using oligonucleotide primers, we amplified by PCR a fragment of the cDNA sequence from a *Drosophila* adult cDNA library (Stratagene). This was used to probe a *Drosophila* embryonic cDNA library, from which full-length cDNAs were isolated and sequenced. The *Drosophila iap* (*diap*) ORF contains three BIR motifs, a RING zinc-finger, and a longer protein sequence element between the BIR domains, which is more characteristic of the human IAPs than either of the baculoviral viral IAPs (Fig. 2).

By using a human/mouse hybrid cell panel (BIOS Labora-

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FIG. 1 Effect of NAIP on cell death induced by serum deprivation, menadione (Sigma) and TNF- α . Viability of *a*, CHO cells deprived of serum in adenovirus-infected cells, and *b*, pooled transformants. *c-h*, Cell death induced by menadione in adenovirus-infected CHO(*c*) and Rat-1 (*e, g*) cells, and in pooled transformants of CHO (*d*) and Rat-1 (*f, h*) cells. Adenovirus-infected and *j*, pooled transformants of TNF- α /cycloheximide-treated HeLa cells. Data are presented as averages of either three independently derived transfected pools or infections.

METHODS. For adenovirus assays an adenovirus encoding LacZ, antisense NAIP (PIAN) or vector alone with no insert were used as controls. Bcl-2 was used as a positive control, and pcDNA alone as a negative control in cell line assays. Cell viability was determined by trypan blue exclusion. To generate a full-length 3.7-kb NAIP construct tagged with the myc epitope (MTG-SP3.7), a 2.5-kb Bsu36I/SaI fragment of NAIP cloned into Bluescript was ligated with a Bsu36I/XhoI fragment of plasmid MTG-SE1.7, the expression vector pcDNA3 containing a 300-bp myc epitope and a 1.7-kb fragment of NAIP. HeLa, CHO and Rat-1 cells were transfected by lipofection (GIBCO BRL) with 8 μ g DNA, and G418 resistant transformants were selected by maintaining the cells in 250 μ g ml⁻¹, 400 μ g ml⁻¹ and 800 μ g ml⁻¹ G418, respectively. All cells were maintained in Eagles medium containing 10% fetal calf serum. For construction of the adenovirus, a 3.7-kb BamHI fragment of NAIP was cloned into the Swal site of the adenovirus expression cosmid pAdex1CAwt¹¹. Production of vectors, purification by double caesium chloride gradient and titre determination was as described previously^{12,13}. For each assay, cells were plated at 5×10^4 ml⁻¹ in triplicate. CHO or Rat-1 cells were treated with menadione for 1.5 h, washed 5 times in PBS, and subsequently maintained in normal media. For serum-deprivation assays, cells were washed 5 times in PBS and maintained in media with 0% fetal calf serum. HeLa cells were treated with 20 units ml⁻¹ TNF- α in combination with 30 μ g ml⁻¹ cycloheximide for 17 h. Apoptosis was confirmed for each trigger by propidium iodide staining. Adenovirus-infected cells were subjected to triggers 36 h after infection. LacZ expression was confirmed histochemically by 5-bromo-4-chloro-3-indoyl- β -D-galactoside (X-gal) as described previously¹⁴. Transcription of PIAN was determined by *in situ* hybridization using the DIG-labelled sense oligonucleotide following the manufacturers protocol (Boehringer Mannheim). The human Bcl-2 clone pB4 (ATCC) was digested with EcoRI and ligated into the EcoRI site of pcDNA3.



tories), we have mapped *hiap-1* and *hiap-2* by PCR to chromosome 11 (data not shown). The high degree of conservation (72% for the overall predicted protein sequence) between HIAP-1 and HIAP-2, as well as the location of both genes on chromosome 11, suggests a gene duplication event. In contrast, XIAP exhibits only 44% and 42% conservation with HIAP-1 and HIAP-2, respectively, close to the 40%, 39% and 36% identity that XIAP shares with Cp-IAP, Op-IAP and DIAP, respectively. NAIP is much more distantly related to the other IAPs, with only 25–30% conservation observed. Moreover, NAIP lacks the RING zinc-finger present in the other baculovirus and human IAPs. Despite this, there is a high level of homology through the BIR domains, notably with HIAP-2, within which NAIP exhibits 58% identity.

Xiap, *hiap-1* and *hiap-2* expression was assessed by northern blot analysis of poly(A)⁺ RNAs from a variety of human tissues, both adult and fetal. The heterogeneity of transcript size (9.0 kb for *xiap*, 6.5 kb for *hiap-1*, and 4.5 kb for *hiap-2*) reflects hetero-

geneity in the lengths of 5' and 3' untranslated regions, as the size of the ORFs ranged from 1.5 kb to 1.8 kb. *Xiap* messenger RNA was observed in all adult and fetal tissues examined except peripheral blood leukocytes (Fig. 3a–c). *Hiap-2* message was present in many fetal and adult tissues, but was most highly expressed in adult skeletal muscle and pancreas, and was low or absent in brain and peripheral blood leukocytes. *Hiap-1* mRNA displayed a unique fetal distribution, in which lung and kidney, but not brain or liver, expressed high levels of the mRNA. In the adult, *hiap-1* mRNA was highly expressed in lymphoid tissues, including spleen, thymus and peripheral blood lymphocytes, suggesting a role in lymphocyte development.

In a manner similar to that described for NAIP, the putative anti-apoptotic activity of XIAP, HIAP-1 and HIAP-2 was assessed in CHO and Rat-1 cells transfected with each of the individual expression plasmids, using CMV-*bcl-2* plasmid as a positive control. Additional constructs included pcDNA3 with the 6-myc tag

METHODS. Probe for the *xiap* cDNA library screening was generated by PCR amplification of a fragment of the X-linked STS (GenBank accession number L24579) product using the primers 5' d-GCAGCTAAGCGCGTGCAGAG and 5' d-GTTCACATCACATTCATCA. PCR amplification of ETS no. T96284 was performed with primers 5' d-CATAATAAAAA-CCCACACTTG and 5' d-CAATTGCGAACCGAAGGATAA. Primers 5' d-CTGTGTCGCCAATGGTTTCTTT and 5' d-GCAGCAATGCCTTGATGTCTG were used to amplify by PCR a cDNA fragment from an adult *Drosophila* cDNA library (Stratagene) corresponding to a fragment of the genomic DNA sequence of the putative *Drosophila iap* gene (GenBank accession number M96581). This probe was then used to screen an embryonic *Drosophila* cDNA library. Protein sequence alignment was accomplished using the PILEUP program of the GCG Sequence Analysis Package (University of Wisconsin Genetics Computer Group). The two BIR motifs of Op-IAP and Cp-IAP were arbitrarily aligned with BIR 2 and 3 of the *Drosophila* and human IAP protein sequences.

By using serum deprivation as a trigger for apoptosis, we found that all three serum human IAPs demonstrated an ability to suppress apoptosis (Fig. 4a). Control negative cell lines exhibited greatly reduced cell viability 48 and 72 h after serum withdrawal (for example, 39% and 10% viability, respectively, for SMN-expressing CHO cells). Treated cells also demonstrated extensive membrane blebbing, DNA fragmentation, and morphological changes characteristic of apoptosis (data not shown). In contrast, IAP-expressing CHO cells maintained a more normal morphology after serum withdrawal, and had increased survival (69–79% viability at 48 h, 20–28% viability at 72 h). Cells expressing BCL-2 displayed slightly greater viability, showing 89% and 43% survival at 48 and 72 h, respectively (Fig. 4a).

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In conclusion, we have documented an anti-apoptotic effect of NAIP in mammalian cells. In addition, immunofluorescence localization indicates that NAIP is expressed in motor, but not sensory, neurons (manuscript in preparation). These findings are in accordance with the protein acting as a negative regulator of motor-neuron apoptosis and, when deficient or absent, contribut-

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BIR 1

Op_iap MTEL.....VME.....ESVRLATFGGEWPLNAPVSAEDLVANGFF.....GTWMEAECDFC
Cp_iapDNVENSIFLSNLMSK.....ANTFELKY.....DLSELVRYMSTSTFPAGVPSVSESLARAGFYITGVNDKVKFCFC
diapMHKTASQRLFPGPSYQNIKSIMEDSTILSDWNTS.....DESELYRMSTYSTFPAGVPSVSESLARAGFYITGVNDKVKFCFC
hiap-1EKVDKSYFOEKMFTNSFEGSKTCVPADINKEE.....EVEFENRLKTEITANFPSPGSVPASVTLARAGELYTGEGDVRFCSC
hiap-2MATQQKASDERISQFDHNLPLPELSALLGLDAVLQAKELEEEQKERAKMQKGYNSMR.....EAKRLKTEVITYEYPSWISOEMAAAGFYITGVKSGIGCFCC
xiapE.....RL-TF-F.....L.....GFY-G.....C-C
naip
consensusE-RL-TF-F-L-GFY-G-C-C

BIR 2

Op_iap MMSRAIGAPOEGA.....MS.....
Cp_iapHVRIDRWEYGLDVAERHRRSSPIGSLAPLNHCGNVPRQSESDNEGNSVVDSPESCSCP.....
diapGLMLDNWKRGDSPTEKRLKLYPSQRFVQSLNSVNNLEATSPQTFPSSVTHS.....THSLLPGETENSGYFRGYSNPSHPNVNSRANG.....EFS
hiap-1GLMLDNWKLGDSPTEKRLKLYPSQRFVQSLNSVNNLEATSPQTFPSSVTHS.....THSLLPGETENSGYFRGYSNPSHPNVNSRANG.....EFS
hiap-2HAADVDRMQYGDQAVGRHRRKRVNRRFTNGFY.....L.....ENSATQSTNSGQNGQYKVENYLSGRDHFALDRPSETHADYLLRTGTQGVVDIS
xiapSLILFGLAGLTRLPIEDHKKRPHEDGFI.....LNKDVGNGIAKYDIRVKNLKSRLGGKMRQYE.....
naipH-P-C
consensusH-P-C

BIR 3

Op_iap DMKKAARLGTITYNWP.....VQFLEPSRMAASGFYLLRGDEVRCAFCQKVEITNVVRGDDPETDHRKWAQCPQVRN.....N.....
Cp_iapDLRLIEVRNLTFEKFWE.....VSFLDSPETMAKNGEYLLGRSDEVRCAFCQKVEIMRWKEGDDPADHKKWAQCPQVRKGIQDVCGSIVT.....
diapDLLLEARNLVEFKDWP.....NPNITPQALAKAGFYLLNRLDHVKCQWNGVIAKWEKNDAWEHRRHFFQCPQVRMGPILEFATG.....
hiap-1ALMRSSYPCPNENARLLTHLTFW.....LTFISPTDLARAGFYIIGPGRDVAFCAGGKLSNWPKPDAWSEHRRHFFQCPQVRMGPILEFATG.....E
hiap-2SSRTNPYSYAMSTDEARLYTHHMMF.....LTFISPTDLARAGFYIIGPGRDVAFCAGGKLSNWPKPDAWSEHRRHFFQCPQVRMGPILEFATG.....E
xiapDTIYPRNP.....AMYCEARLKSQNW.....DYAHUTPRELASAGLYTIGDGVQFCGCGKLNWECPRDASHEHRRHFFQCPQVRMGPILEFATG.....E
naipEAPRLASRNWFFYVQGISPCVLEAGFVFTGKQDVTQVCFSGGCLNWEQDDPWKEHAKWEPKCEFI.....RKSKESEETQYIQSYGK
consensusM-E-ARL-TF-WP-L-P-LA-AGFY-G-D-V-C-C-G-NWE-D-EH-R-FP-CPFY

BIR 4

Op_iap AHDTPHDPAPPARSAAHQPYAT.....EAAARLRTFAEWPRGLKQRFPELAEAGFYITGGGKTRCFCDGGGLKDWPEDDAPWQOAHARWYDRGEYV
Cp_iapTNNTQNTTTHDTIIGPAHPKYAT.....EAAARLRTFAEWPRGLKQRFPELAEAGFYITGGGKTRCFCDGGGLKDWPEDDAPWQOAHARWYDRGEYV
diapKNLDELGIQPTTL.....PLRPKYACVDARLRTFDWPISNIQPASALQAGLYYQKIGDQVRFCPHCNIGLRSWQKEDEEPWEHAKWSKQCFV
hiap-1NQLQDSTRYTVS.....NLSMGTAAAREKTFEFPWSSVLVNPQOLASAGFYIYGNSSDDVKFCGCGDGLRWESGDDPWFQAHAKWFRCEGL
hiap-2NSL.....ETLRFIS.....NLSMGTAAAREKTFEFPWSSVLVNPQOLASAGFYIYGNSSDDVKFCGCGDGLRWESGDDPWFQAHAKWFRCEGL
xiapDAVSSDRNFPNSTNLPRNPSMAYEARIFTFTGWIYS.....VNKEOLARAGELYEGGKVKCFHCGGGLTDWKPSEDDPWEHAKWFRCEGL
naipFVDITGEHFVNSVWQRELPMASAYCNDISFI.....EELRLDSFKQWPRESAVGVAALAKAGLEYTGKIDVQCFSGGCLNWEQDDPWKEHAKWFRCEGL
consensusA-ARL-TF-WP-E-LA-AGFY-G-D-V-C-C-GGL--D--DDPW-HA-WFP-C-Y

BIR 5

Op_iap LLVKGREDVQVRMT.....EACVVRDA.....
Cp_iapQLVKGREDVQVRMT.....EACVVRDA.....
diapLRAIKGRDYVQVRMT.....EACVVRDA.....
hiap-1LRAIKGRDYVQVRMT.....EACVVRDA.....
hiap-2LRAIKGRDYVQVRMT.....EACVVRDA.....
xiapLRAIKGRDYVQVRMT.....EACVVRDA.....
naipLRAIKGRDYVQVRMT.....EACVVRDA.....
consensusKG-E-V-V-V

BIR 6

Op_iap DN.....EPHIERPAV.....
Cp_iapNTTVSTAAPVSEPIPETKIEK.....
diapELLHDIFDDAGAGAALEVREPEPSAP.....FIEPCQATTSKAASVPIPADSIAPKPAQ.....
hiap-1DVLVDLNAEDEIRREEERATEEESNDLLLRKNRMLFQHLTCVPIPLDLSLITAGINEQHDVIKQKTQSLQARELIDTLVKGNTATVF
hiap-2DIVALLNAEDEIRREEERATEEESNDLLLRKNRMLFQHLTCVPIPLDLSLITAGINEQHDVIKQKTQSLQARELIDTLVKGNTATVF
xiapVLVADLVNAQDKQDE.....SSQSLQ.....
naipHISKPVQEPVLVPEVGNLNSVMVCEAGSGKTLRLKIAFLWASGSCPLLNRQLVFLVLSLSTRQDEGLASIQDLLEKSGSVNEMCRNIQQLK
consensusR-KVEM-E-V-F-PCGH-V-C-CA-CP-CR-I-FLS

RZF

Op_iap EAEVADRICKICLGAETKVCVPCGHVACCKAGCAVYITCPVCRGOLDKAVRMVYV.....
Cp_iapEPOVEDSKIKICVLEECVCPVPCGHVACCKAGCAVYITCPVCRGOLDKAVRMVYV.....
diapAEAVSNISKITDIEIKMSVSTPNGLNLEENRQLDARLCKVCEDEGVVFLPCGHALTNCQCAPSVNACRAIDKGFVTFELS
hiap-1RNSLQEAEAVALYEHFVQQDIKIYIPTEVDVSLPVEEQLRLPEERTCKVCMKDEVSIVFIPCGHVLVCKDCAPSRLKPCICSTIKGTVRTFELS
hiap-2KNCLKEISTSLYKNNLFDVDMNKYIPIEDVSGLSLEQLRLQERCTCKVCMKDEVSIVFIPCGHVLVCKDCAPSRLKPCICSTIKGTVRTFELS
xiapNQVFLFLDDYKEICSIPOVIGFKLQKNHLSRTCLLAVITRNRRARDIRYLELITELIQAFPPYITVCLIRLHSHNMLRLKFMFYFGKNSLQIKQKTPFL
naipR-KVEM-E-V-F-PCGH-V-C-CA-CP-CR-I-FLS
consensusR-KVEM-E-V-F-PCGH-V-C-CA-CP-CR-I-FLS

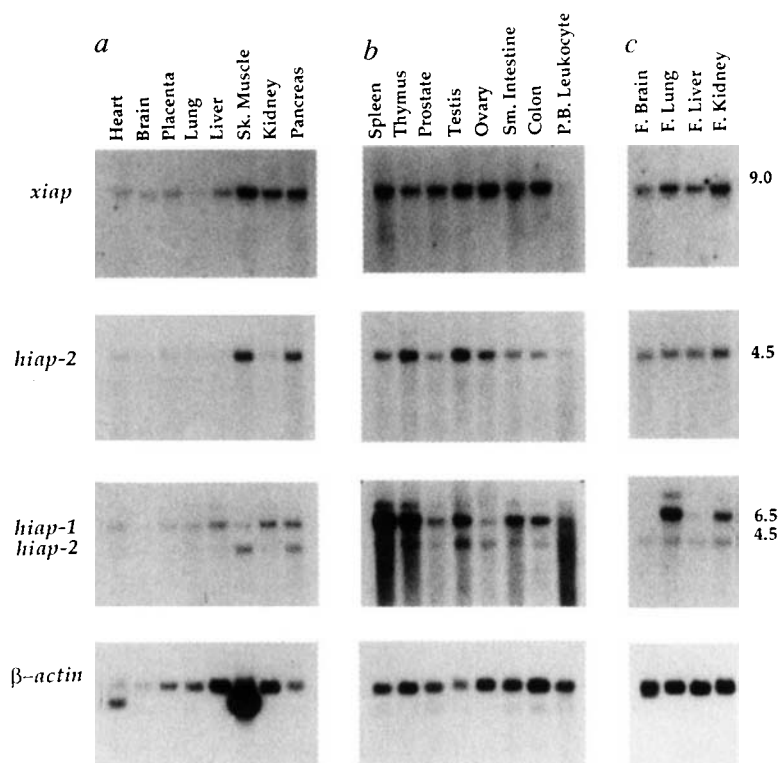


FIG. 3 Northern blot analysis of the human *iap* genes. **a**, Human multiple-tissue northern blot (Clontech MTN blot I) containing 2 μ g per lane poly(A)⁺ RNA probed sequentially with [³²P]dCTP (Amersham) labelled, random-primed (Amersham Rediprime) DNA probe using: (1) a 1.6-kb fragment spanning the entire *xiap* coding region; (2) a 375-bp *hiap-2*-specific probe from the 3' untranslated region; (3) a 1.3-kb fragment from the *hiap-1* coding region spanning two of the BIR domains and the RING-zinc-finger domain that cross-hybridizes with both *hiap-1* and *hiap-2*; and (4) control β -actin cDNA probe provided by the manufacturer. Blots were hybridized overnight in 5X SSPE/10X Denhardt's solution/100 mg ml⁻¹ salmon sperm DNA/50% formamide/2% SDS containing 1.5×10^6 c.p.m. m⁻¹ probe DNA. Blots were washed twice with 2X SSC/0.1% SDS for 15 min at room temperature, followed by 1 wash with 2X SSC/0.1% SDS at 50 °C. Membranes were exposed to X-Omat AR film (Kodak) at -70 °C using 'L Plus' intensifying screens (Fisher). **b**, Human multiple tissue northern blot containing 2 μ g per lane Poly(A)⁺ RNA (MTN blot II, Clontech) probed sequentially as in **a**. **c**, Human fetal multiple tissue northern blot (Clontech) containing 2 μ g per lane poly(A)⁺ RNA probed as in **a** and **b**. Sk., skeletal; Sm., small; P.B., peripheral blood; F., fetal.

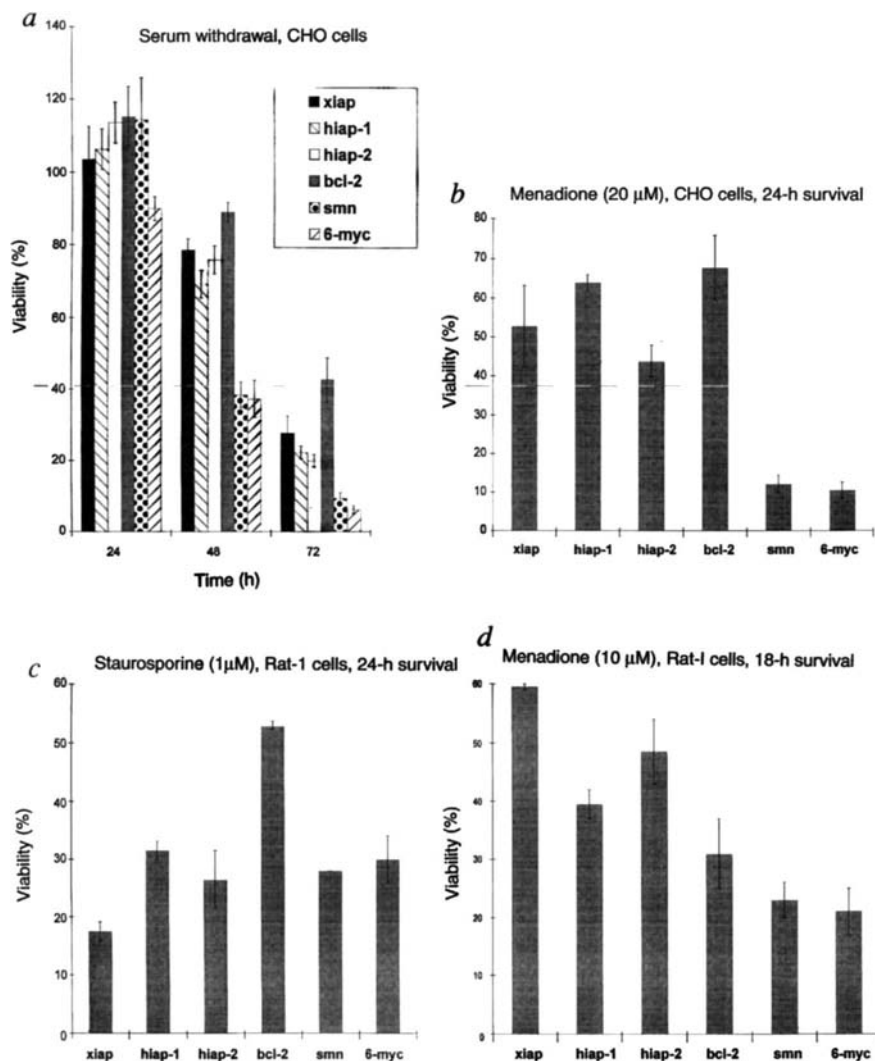


FIG. 4 Apoptosis-suppression assays. **a**, Survival of CHO cells after withdrawal of serum. **b**, Survival of CHO transfected cell lines after exposure to menadione. **c**, Survival of Rat-1 cells after exposure to staurosporine. **d**, Survival of Rat-1 cells after exposure to menadione.

METHODS. CHO cells were transfected by Lipofectate with 2 μ g of each of the following recombinant plasmids: pcDNA3-6myc-*hiap-1*, pcDNA3-6myc-*hiap-2*, pcDNA3-6myc-*xiap*, pcDNA3-6myc, pcDNA3-HA-*smn*, and pcDNA3-*bcl-2*. Oligonucleotide primers were synthesized to allow PCR amplification and cloning of the *xiap*, *hiap-1* and *hiap-2* ORFs in pcDNA3 (Invitrogen). Each construct was modified to incorporate a synthetic myc tag encoding six repeats of the peptide sequence MEQK-LISEEDL allowing detection of myc-IAP fusion proteins by monoclonal anti-myc antiserum¹⁵. In **a**, triplicate samples of cell lines in 24-well dishes were washed 5 times with serum-free media and maintained in serum-free conditions during the course of the experiment. Trypan blue exclusion counting of viable cells using a haemocytometer was performed on samples at time 0, and 24, 48 and 72 h after serum withdrawal. Survival was calculated as a percentage of initial numbers. Numbers represent the average of three separate experiments performed in triplicate, $\pm$ average deviation. In **b**, cell lines were plated in 24-well dishes, allowed to grow overnight, then exposed for 1.5 h to 20 μ M menadione (Sigma). Triplicate samples were collected at the time of exposure and 24 h after exposure, and assessed by trypan blue exclusion for survival. Data represent the average of three independent experiments, $\pm$ average deviation. In **c**, Rat-1 cells were transfected with the same plasmids as in **a**, with selection in 800 μ g ml⁻¹ G418 media for two weeks. Cell lines were assessed for resistance to 1 μ M staurosporine induced apoptosis for 5 h. Viable cell counts were obtained 24 h after exposure by trypan blue exclusion counting of samples prepared in triplicate. Numbers represent the average of two independent experiments, $\pm$ average deviation. In **d**, Rat-1 cell lines were tested for resistance to 10 μ M menadione for 1.5 h, then counted 18 h after exposure. Numbers represent the average of three independent experiments performed in triplicate, $\pm$ average deviation.

ing to the SMA phenotype. Such a model does not explain the role of SMN in SMA, as we have been unable to demonstrate an anti-apoptotic effect with the SMN protein. Nonetheless, the genetic evidence for SMN involvement in SMA is strong, and it may be that the absence of SMN results in a pathological effect which is additive to that of NAIP deficiency. We have also identified a human multi-gene family which shows homology to baculoviral IAPs, and which encode proteins that demonstrate significant inhibition of apoptosis. It remains to be determined how the IAPs fit into the apoptotic cascades that are now being characterized. The conservation of IAPs from humans to *Drosophila* suggests that they are important for the regulation of apoptosis. Our

finding that the IAPs function under different but overlapping circumstances with respect to BCL-2 is consistent with the existence of several parallel pathways for inducing and suppressing programmed cell death. Although the repertoire of proteins involved in the initiation and/or execution of apoptosis has greatly expanded in recent years⁸⁻¹⁰, inhibitors of apoptosis remain relatively rare by comparison, the most prominent of which remains the Bcl-2 family. We believe that the identification of *naip* and this new family of human *iap* genes will significantly increase our understanding of both the biological processes involved in programmed cell death and diseases associated with dysregulation of apoptosis. □

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A presynaptic inositol-5-phosphatase

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SYNAPTOJANIN is a nerve terminal protein of relative molecular mass 145,000 which appears to participate with dynamin in synaptic vesicle recycling^{1,2}. The central region of synaptojanin defines it as a member of the inositol-5-phosphatase family, which includes the product of the gene that is defective in the oculocerebrorenal syndrome of Lowe³⁻⁷. Synaptojanin has 5-phosphatase activity and its amino-terminal domain is homologous with the yeast protein Sac1 (Rsd1), which is genetically implicated in phospholipid metabolism and in the function of the actin cytoskeleton⁸⁻¹⁰. The carboxy terminus, which is of different lengths in adult and developing neurons owing to the alternative use of two termination sites, is proline-rich, consistent with the reported interaction of synaptojanin with the SH3 domains of Grb2 (refs 1, 2). Synaptojanin is the only other major brain protein besides dynamin that binds the SH3 domain of amphiphysin, a presynaptic protein with a putative function in endocytosis¹¹⁻¹⁴. Our results suggest a link between phosphoinositide metabolism and synaptic vesicle recycling.

Peptide sequences from purified synaptojanin² were used to design oligonucleotides for amplification by polymerase chain reaction (PCR) from rat brain cDNA. The isolated DNA fragment hybridized to a single 7.5-kilobase (kb) messenger RNA

highly enriched in brain (data not shown) and was used as a probe to isolate overlapping clones from two rat brain cDNA libraries, including a full-length clone (clone 9). The amino-acid sequence of synaptojanin determined from clone-9 is shown in Fig. 1a. The coding sequence begins at nucleotide 143, with the first methionine following an in-frame stop codon. This methionine, which is within a good Kozak consensus sequence¹⁵ is followed by a 3,876-base-pair (bp) open reading frame (ORF1) ending with a UAA stop codon at position 4,019. ORF1 encodes a putative protein of 1,292 amino acids with a predicted isoelectric point of 6.96, a predicted relative molecular mass of 142,797 and no putative transmembrane regions. It contains all three peptide sequences obtained from the adult rat brain protein and a region rich in prolines at its C terminus (amino acids 1,042–1,292), with several consensus sites for SH3-domain interactions¹⁶. Sequencing of three other clones revealed an identical amino-acid sequence, with the exception of a 16-amino-acid insert which probably results from alternative splicing (arrowheads in Fig. 1a).

Surprisingly, after the UAA stop codon the cDNA predicts a second in-frame ORF (ORF2) of 798 nucleotides that encodes an additional proline-rich sequence of 266 amino acids (*M*_r 28,374 with consensus SH3-binding domains¹⁶. ORF2 ends with two successive stop codons at nucleotide positions 4,820 and 4,823. The UAA stop codon at nucleotide position 4,019 was present in multiple clones and in a partial clone of human synaptojanin. Further, COS cells express a 145K protein comigrating with brain synaptojanin and recognized by anti-synaptojanin antibodies when they are transfected with cDNAs encoding either ORF1 alone or ORF1 followed by ORF2 (Fig. 1b).

Extracts of neonate brain and undifferentiated PC-12 cells contain, in addition to the 145K band, a band of 170K, which is enriched by Grb2 affinity chromatography, is recognized by antibodies raised against the 145K band from rat brain² and selectively by an anti-peptide antibody raised against sequences specific to ORF2 (Fig. 1c). Thus, the 170K protein represents a longer form of synaptojanin containing both ORF1 and ORF2. Possible mechanisms for the suppression of the stop codon to generate the 170K band include readthrough¹⁷, alternative splicing¹⁸ or RNA editing¹⁹.

A search of databases revealed two blocks of homology with other proteins. The N-terminal domain is similar to the cytosolic domain of the yeast Sac1 (Rsd1) protein (Fig. 2a). The *SAC1* gene was identified in screens for suppressors of an actin mutant (*act1-1*)⁹ and of the *sec14* mutant¹⁰, which is defective in post-Golgi

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membrane trafficking²⁰. The Sec14 protein is a phosphatidylinositol/phosphatidylcholine transfer protein²¹. Strikingly, yeast *sac1* strains are inositol auxotrophs that require elevated levels of inositol for growth¹⁰. Furthermore, *sac1* mutant alleles are capable of suppressing other secretory mutants including *sec9* and *sec6* (ref. 8).

The central region of synaptojanin is similar to a family of 5-phosphatases that dephosphorylate inositol polyphosphates and inositol phospholipids at the 5' position of the inositol ring (Fig. 2). The homology is particularly strong in a region (amino acids 780–868 of synaptojanin) that is thought to mediate enzyme activity⁶ (Fig. 2b). This family includes type I and type II 5-phosphatases^{5–7} as well as the product of the gene defective in the oculocerebrorenal syndrome of Lowe (OCRL)³, which functions as a type-II 5-phosphatase⁴. Finally, two ORFs of unknown function that include both the Sac1 and the 5-phosphatase homology domains of synaptojanin are present in yeast databases.

Synaptojanin was suggested to participate with dynamin in

synaptic vesicle endocytosis and recycling^{1,2}. We have shown that the proline-rich C terminus of dynamin interacts *in vitro* and in brain extracts with the SH3 domain of amphiphysin¹⁴, a nerve terminal protein¹¹ that also interacts through a distinct domain with the clathrin adaptor AP2 (refs 13, 14) and may therefore help concentrate dynamin at clathrin-coated pits. Amphiphysin contains regions of similarity to the proteins encoded by the *RVS161* (allelic to *END6*) and *RVS167* yeast genes¹¹, deletions in which produce endocytosis defects¹². Amphiphysin colocalizes with synaptojanin in brain sections (Fig. 3a), and, like dynamin¹⁴, binds the SH3 domain of amphiphysin (Fig. 3b). In fact, when a total brain extract was incubated with a glutathione *S*-transferase (GST) fusion protein comprising the SH3 domain of amphiphysin (GST/amphiphysin (SH3)), synaptojanin was the only other major protein besides dynamin¹⁴ (Fig. 4b) to be retained specifically by the column. Synapsin I, which like dynamin and synaptojanin binds the SH3 domains of Grb2 (refs 1, 2), did not bind¹⁴ (Fig. 3b). None of these three proteins binds to the SH3 domain of SAP90

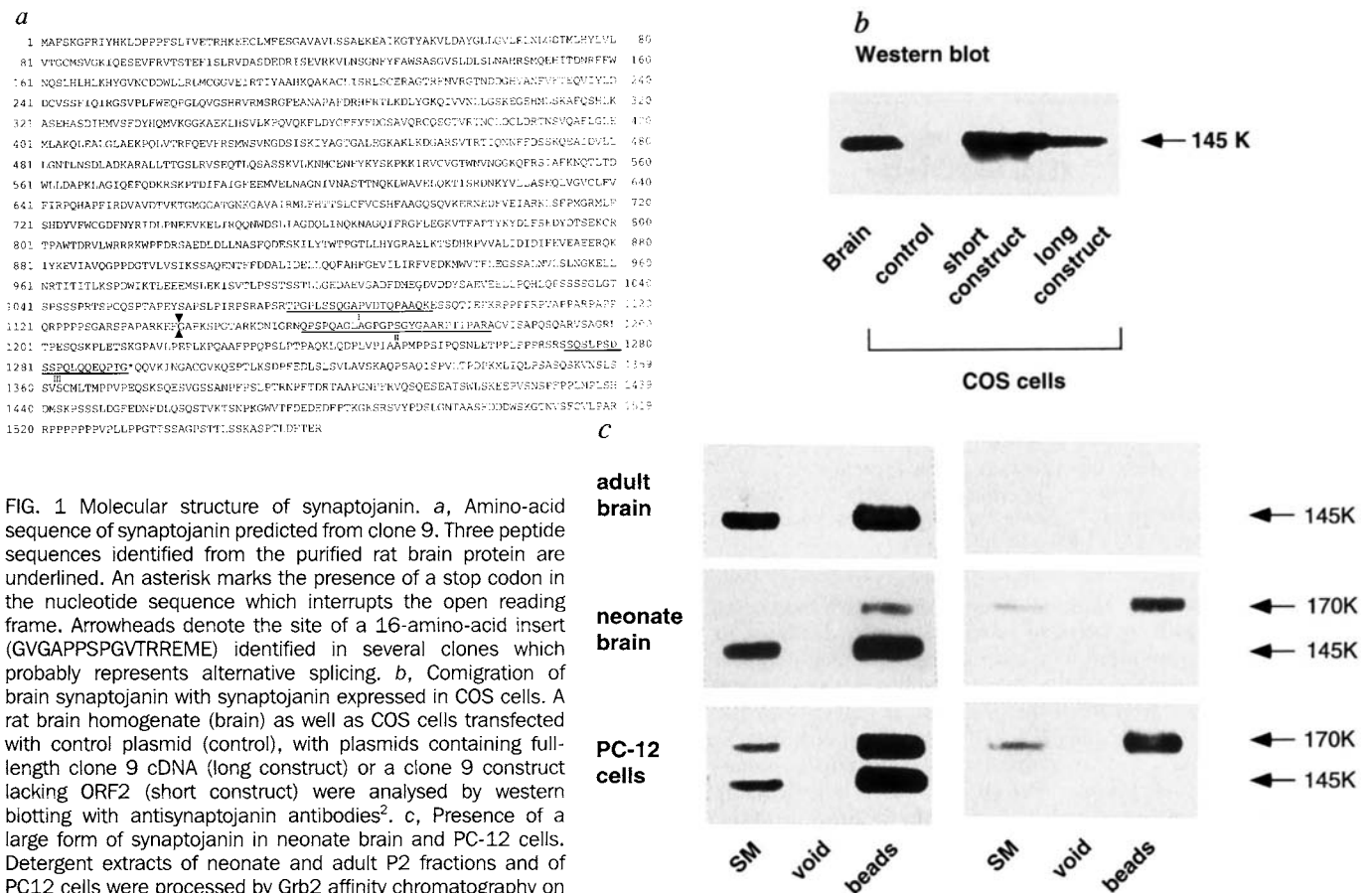
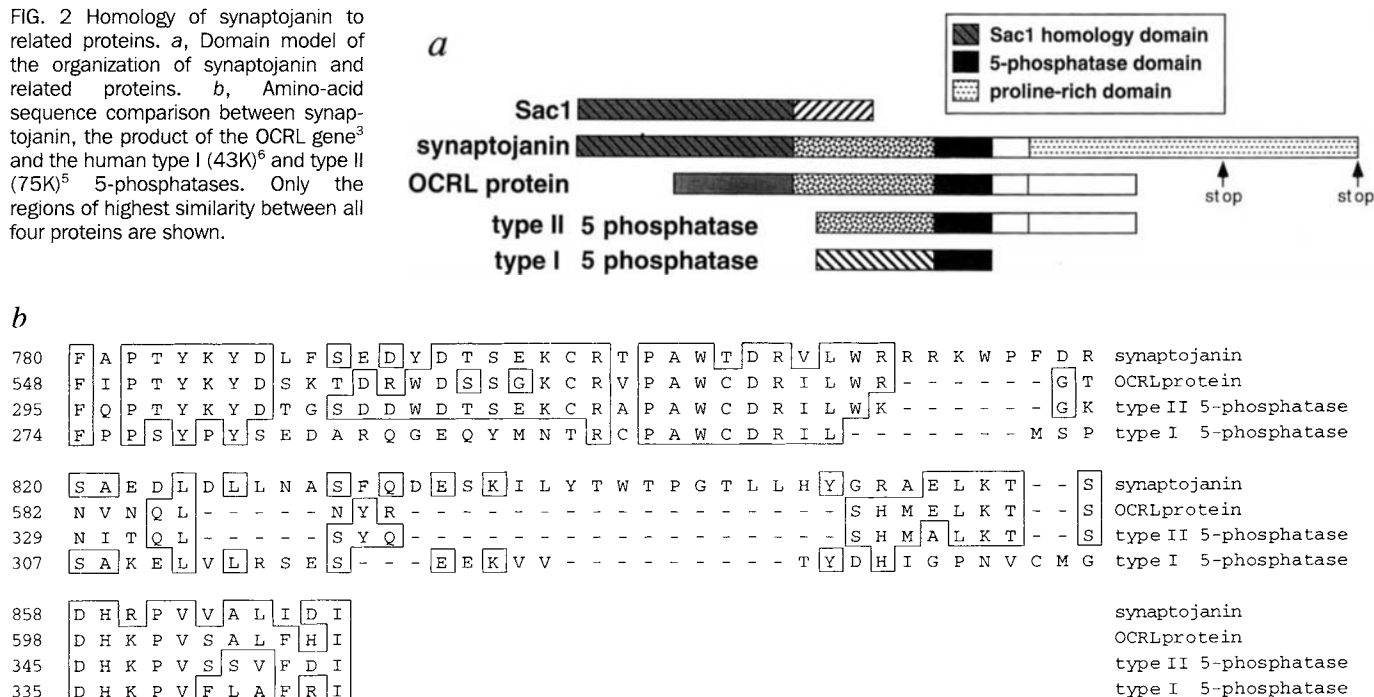


FIG. 1 Molecular structure of synaptojanin. **a**, Amino-acid sequence of synaptojanin predicted from clone 9. Three peptide sequences identified from the purified rat brain protein are underlined. An asterisk marks the presence of a stop codon in the nucleotide sequence which interrupts the open reading frame. Arrowheads denote the site of a 16-amino-acid insert (GVGAPPSPGVTRREME) identified in several clones which probably represents alternative splicing. **b**, Comigration of brain synaptojanin with synaptojanin expressed in COS cells. A rat brain homogenate (brain) as well as COS cells transfected with control plasmid (control), with plasmids containing full-length clone 9 cDNA (long construct) or a clone 9 construct lacking ORF2 (short construct) were analysed by western blotting with antisynaptojanin antibodies². **c**, Presence of a large form of synaptojanin in neonate brain and PC-12 cells. Detergent extracts of neonate and adult P2 fractions and of PC12 cells were processed by Grb2 affinity chromatography on GST/Grb2 (refs 1, 2), and material specifically bound to the beads was eluted with SDS-PAGE sample buffer. Immunoblots are shown of equal aliquots of starting material (SM), unbound (void) and bound material (Grb2 beads) processed by western blotting with (left panels) affinity-purified antibody against the 145K form of synaptojanin² or (right panels) with an anti-peptide antibody against sequences specific to ORF2 (WSKGTNVSFCVLPAR; CVLPARRPPPPPPVP; STTLSSKASPTLDFTER). **METHODS.** Preparation by PCR of a synaptojanin probe: Synaptojanin was isolated from rat brain² and internal amino-acid sequences were determined from gel slices at the Yale/Keck Biotechnology Facility. Three peptide sequences (I–III) were identified as indicated in **a**. Two degenerate oligonucleotide primers (QPSPQA and GYGAAR) from peptide II were used for PCR from rat brain cDNA. A DNA product of the expected size (60 bp) was subcloned, sequenced, and found to encode the peptide QPSPQA-GLAGPGPSGYGAAR. Non-degenerate forward and reverse PCR primers were synthesized based on this sequence and were used in PCR reactions

from rat brain cDNA with forward and reverse degenerate primers (QEQPTG) from peptide II. A product of 386 bp was subcloned, sequenced, and found to encode sequences from peptides II and III. The PCR fragment was radiolabelled and used for northern analysis and library screening. Cloning of synaptojanin: The labelled PCR product was used to screen $\sim 1.25 \times 10^6$ phage plaques from an oligo(dT)-primed λ -ZAP II rat brain cDNA library (Stratagene, no. 936501). Three positive clones were plaque-purified and sequenced. Probes based on these sequences were used to isolate a full-length clone from a >4 -kb size-selected cDNA library (gift from T. Snutch). The sequence of the full-length clone 9 is shown. COS cell transfections: The long construct was made by cloning the *EcoRI* fragment from clone 9 into the *EcoRI* site of pcDNA3. The short construct was made by replacing the DNA fragment encoding nucleotides 3,967–5,971 of clone 9 in pcDNA3 with a PCR-generated fragment that terminates at nucleotide 4,169, thus deleting ORF2.

FIG. 2 Homology of synaptojanin to related proteins. *a*, Domain model of the organization of synaptojanin and related proteins. *b*, Amino-acid sequence comparison between synaptojanin, the product of the OCRL gene³ and the human type I (43K)⁶ and type II (75K)⁵ 5-phosphatases. Only the regions of highest similarity between all four proteins are shown.



(Fig. 3b), an SH3 domain-containing protein enriched in the submembranous cytoskeleton of the synapse²². These observations confirm the specificity of the interaction of synaptojanin and dynamin with amphiphysin and strengthen the putative link between dynamin and synaptojanin function².

To prove that synaptojanin is a 5-phosphatase, as suggested by its primary sequence, we tested the enzyme activity of the purified protein. Anti-synaptojanin immunoprecipitates obtained from adult brain extracts converted the tritiated inositol trisphosphate [³H]Ins(1,4,5)P₃, but not [³H]Ins(1,3,4)P₃, to inositol bisphos-

phate, [³H]InsP₂ (Fig. 4a). These results were confirmed by analysis of the purified protein. The eluate from the GST/amphiphysin(SH3) column (Fig. 4b, left lane) was subjected to gel filtration under transient denaturing conditions to separate dynamin and synaptojanin from GST/amphiphysin(SH3); synaptojanin was then further purified by anion-exchange chromatography, where synaptojanin co-eluted exactly with the inositol-5-phosphatase activity. This material was 220-fold enriched in specific activity over the starting material. Synaptojanin used both Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ as substrates and

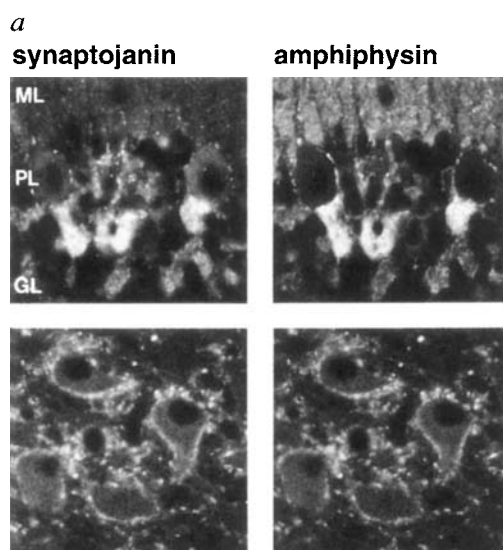
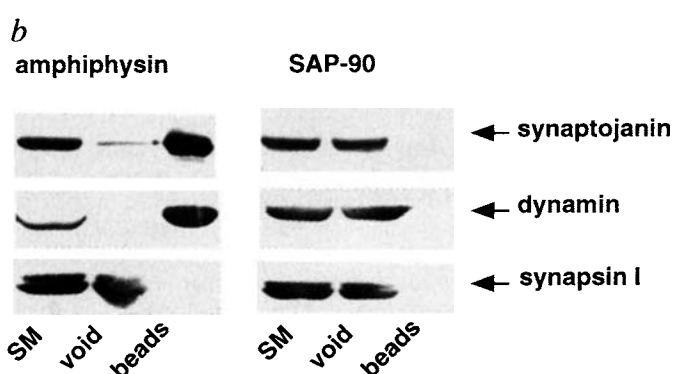


FIG. 3 Interaction of synaptojanin with amphiphysin. *a*, Double immunofluorescence micrographs demonstrating colocalization of synaptojanin and amphiphysin in frozen sections of rat brain. Top, section of the cerebellar cortex: molecular layer (ML), Purkinje cell layer (PL), granule cell layer (GL). Bottom, section of the brainstem. In both fields immunoreactivity is concentrated in nerve terminals, including the large terminals



that surround axon hillocks of Purkinje cells. *b*, Specific interaction of synaptojanin with the SH3 domain of amphiphysin. An extract of adult rat brain was incubated with GST/amphiphysin(SH3) or SAP-90/GST coupled to glutathione-Sepharose, and bound material eluted with SDS-PAGE sample buffer. Immunoblots are shown for the proteins indicated of aliquots of starting material (SM), unbound (void), and bound material (beads).

METHODS. Immunofluorescence: Double fluorescence immunostaining of rat brain sections was performed by standard methods with a rabbit antiserum against amphiphysin¹¹ and a mouse antiserum against amino acids 1,156–1,286 of synaptojanin. SH3 domain affinity chromatography: proteins from an adult rat brain P₂ fraction² were affinity-purified on GST/amphiphysin(SH3) or GST/SAP-90 fusion protein²² as described^{1,2}.

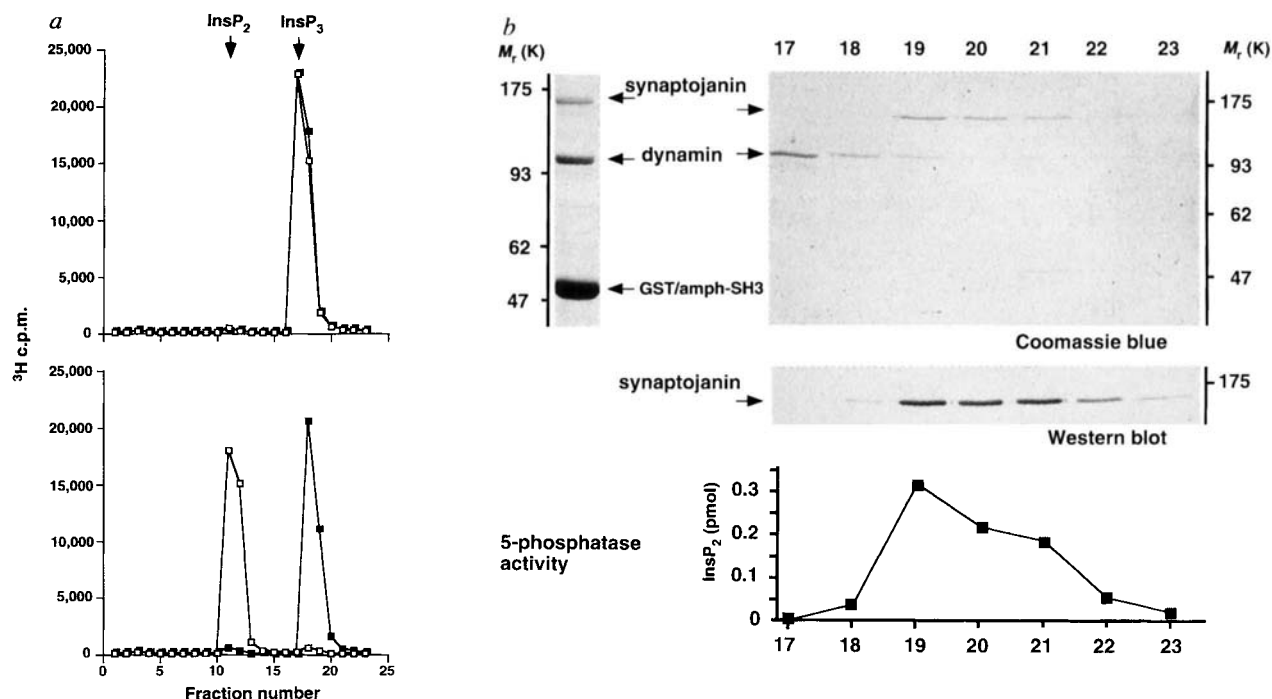


FIG. 4 Synaptojanin has 5-phosphatase activity. **a**, Dephosphorylation of [^3H]InsP₃ by synaptojanin immunoprecipitates. Protein A-Sepharose immunoprecipitates obtained by affinity-purified anti-synaptojanin antibody (open squares) or control rabbit IgG (filled squares) were incubated with [^3H]Ins(1,3,4)P₃ (top) or [^3H]Ins(1,4,5)P₃ (bottom) and the products separated by FPLC. Positions indicated for InsP₃ and InsP₂ were determined using purified compounds run separately. **b**, Copurification of synaptojanin and inositol-5-phosphatase activity. Synaptojanin was purified from a rat brain extract by a three-step procedure involving in sequence: affinity chromatography on GST/amphiphysin(SH3), gel filtration and ion exchange chromatography. The left hand gel lane shows protein from the eluate from the GST/amphiphysin(SH3) beads. Lanes 17–23 are the fractions from the anion-exchange column containing the peak of synaptojanin and inositol-5-phosphatase activity. Note the identical elution patterns of enzyme activity and of synaptojanin as visualized by protein staining and western blotting.

METHODS. Immunoprecipitation: Rat brain cytosol (15 mg) was incubated with 10 μg of affinity-purified anti-synaptojanin antibody² or control rabbit IgG in the presence of 0.5% Triton X-100 and 150 mM NaCl overnight at 4 °C before the addition of 20 μl of protein A-Sepharose for 3 h. The beads

were washed extensively in Tris-buffered saline containing 2 mM MgCl₂ and 0.5% Triton X-100. Phosphatase activity was assayed as described⁴. Briefly, immunobeads were incubated with 0.1 μCi of [^3H]Ins(1,3,4)P₃ or [^3H]Ins(1,4,5)P₃ for 30 min at 37 °C in 20 mM Tris-Cl, pH 7.4, 100 mM NaCl, 2 mM MgCl₂; the products were separated by FPLC on a Waters Protein-Pak Q-8HR (5 $\times$ 50) column. The column was eluted with a 20-ml linear gradient (20–800 mM) of NH₄H₂PO₄, 0.6 ml fractions were collected, and radioactivity determined by liquid scintillation counting. Synaptojanin purification: NP40-solubilized rat brain homogenate¹⁴ was incubated with GST/amphiphysin(SH3) coupled to glutathione-Sepharose and the bound proteins were eluted with 5 mM glutathione, concentrated and loaded onto a Superdex-200 gel filtration column (Pharmacia) after the application to the column of half a void volume of 5M urea in column buffer (20 mM Tris-HCl, pH 7.4, 0.3 M NaCl, 2 mM MgCl₂, 1 mM DTT). Fractions containing synaptojanin and dynamin were pooled, dialysed and applied to a Protein-Pak Q-8HR column. The column was eluted with a 20-ml linear gradient (0–300 mM NaCl in Tris-HCl, pH 8.0, 2 mM MgCl₂, 1 mM DTT) and 0.7-ml fractions were collected. Western blot analysis was carried out with affinity-purified anti-synaptojanin antibodies using alkaline phosphatase for antibody detection.

also dephosphorylated phosphatidylinositol-(4,5)bisphosphate (PtdInsP₂) to phosphatidylinositol phosphate, as determined by thin-layer chromatography (data not shown).

The finding that synaptojanin comprises two domains which are linked to inositol phosphate metabolism (thus the name from the Roman god with two faces, Janus) suggests that inositol metabolism has an important role in nerve terminal function. Growing evidence indicates a direct role of inositol-containing metabolites in the synaptic vesicle cycle. Phosphoinositides and/or inositol polyphosphates bind *in vitro* to, and regulate, proteins implicated in synaptic vesicle cycling including synaptotagmin^{23,24}, the clathrin adaptors AP2 (ref. 25) and AP3/AP180 (refs. 26, 27), and dynamin (I. Gout and M. Waterfield, personal communication). Furthermore, a PtdIns/Ptd-choline transfer protein and a type-1 phosphatidylinositol-4-phosphate-5-OH kinase function in Ca²⁺-regulated exocytosis from permeabilized neuroendocrine cells^{28,29}. To our knowledge, our results show for the first time that a key enzyme of phosphoinositide metabolism is highly concentrated at the nerve terminal, a site where these interactions presumably occur, and provide strong support for their participation in synaptic vesicle recycling. They are convergent with the growing evidence indicating an important general role of phosphoinositides in vesicular traffic^{29,30}. As synaptojanin is a major

Grb2-binding protein of the brain^{1,2}, it may have additional functions in inositol polyphosphate second messenger pathways of the nerve terminal. □

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Role for a *Xenopus* Orc2-related protein in controlling DNA replication

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THE SIX-SUBUNIT origin recognition complex (ORC) is essential for the initiation of DNA replication at specific origins in the budding yeast *Saccharomyces cerevisiae*^{1–9}. An important issue is whether DNA replication in higher eukaryotes, in which the characteristics of replication origins are poorly defined¹⁰, occurs by an ORC-dependent mechanism. We have identified a *Xenopus laevis* Orc2-related protein (XORC2) by its ability to rescue a mitotic-catastrophe mutant of the fission yeast *Schizosaccharomyces pombe*. We show that immunodepletion of XORC2 from *Xenopus* egg extracts^{11–13} abolishes the replication of chromosomal DNA but not elongation synthesis on a single-stranded DNA template. Indirect immunofluorescence indicates that XORC2 binds to chromatin well before the commencement of DNA synthesis, and even under conditions that prevent the association of replication licensing factor(s) with the DNA. These findings suggest that Orc2 plays an important role at an early step of chromosomal replication in animal cells.

We have been looking for *Xenopus* regulators of the cell cycle by using a genetic complementation strategy in *S. pombe*. To identify potential negative regulators of the cell cycle, we expressed a frog oocyte complementary DNA library in the yeast strain *mik1::ura4⁺ weel-50 leu1-32* (Fig. 1a)¹⁴. This strain has no inhibitory, Cdc2-specific tyrosine kinase activity at the restrictive temperature, and therefore dies as a result of mitotic catastrophe, the premature entry into M phase¹⁵. One rescuing plasmid (pC337) was found to contain a full-length cDNA with an open reading frame of 558 amino acids (*M_r* 62,887) that displays significant homology to the *S. cerevisiae* Orc2 protein (Fig. 1b)^{3–5}. The XORC2-expressing mutant yeast divided at approximately a wild-type size, showing neither an obvious *wee* nor elongated phenotype. However, the rescued cells, like the original mutant strain, entered mitosis inappropriately in the presence of 12 mM hydroxyurea at the restrictive temperature (not shown). Therefore, although XORC2 formally acts as a negative regulator of the

cell cycle in fission yeast, its overexpression is not sufficient to restore the replication checkpoint.

The primary sequence of XORC2 shares 23% identical residues with the *S. cerevisiae* Orc2 protein; the greatest similarity occurs in the carboxy-terminal half of the protein (29% identical residues). Notably, the XORC2 protein contains 14 Ser–Pro or Thr–Pro motifs, six of which match the consensus for phosphorylation by a cyclin-dependent kinase (CDK). Of these motifs, 12 are concentrated in the amino-terminal half of the protein. XORC2 also contains a putative bipartite nuclear localization sequence (RKNEFISTTPHRLRKR, residues 149–164) that overlaps one potential CDK phosphorylation site.

The replication of sperm chromatin in *Xenopus* egg extracts represents a valuable system for studying the biochemical mechanisms underlying the semiconservative replication of eukaryotic chromosomes^{11–13}. To study the functional properties of XORC2 in this system, we prepared polyclonal antibodies against a histidine-tagged version of XORC2 that had been expressed in baculovirus-infected insect cells (Fig. 2a). These antibodies react strongly with a single polypeptide of *M_r* 63K in S-phase extracts from *Xenopus* eggs (Fig. 2b). XORC2 is present at equivalent amounts in M-phase egg extracts, but the protein appears to be modified at this time, as indicated by a reduced electrophoretic mobility (Fig. 2c). Asynchronous *Xenopus* tissue culture (XTC) cells also contain the 63K form of XORC2 (Fig. 2b), indicating that XORC2 is present in both embryonic and somatic cells. The concentration of XORC2 is 7 ng μl^{−1} (100 nM) in egg extracts and approximately 10-fold lower in XTC cells. In *Xenopus* eggs, the concentration of replication protein A (125 nM)¹³ is comparable to XORC2, whereas proliferating cell nuclear antigen is considerably more abundant (3 μM)¹⁶. Thus, like these replication proteins, XORC2 is stockpiled for the rapid cycles of DNA replication during early development.

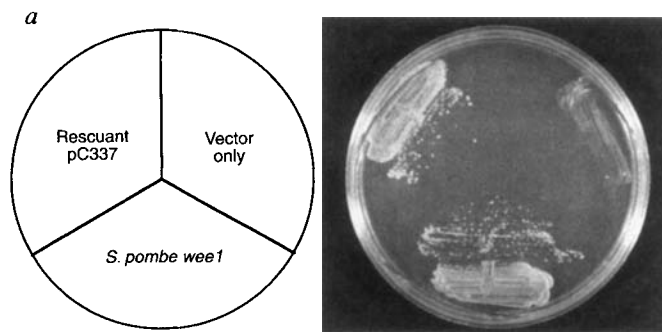
To investigate the role of XORC2 in DNA replication in *Xenopus* egg extracts, we performed a series of immunodepletion experiments (Figs 2d and 3). For this purpose, we treated interphase extracts with protein A beads containing either anti-XORC2 or control antibodies. This procedure removed 90–99% of XORC2 from the anti-XORC2-treated extracts (Fig. 2d). Because formation of the nuclear envelope around sperm chromatin is essential for replication^{11,12}, we verified that the XORC2-depleted and control extracts were equally efficient in supporting nuclear assembly (Fig. 2e). Finally, we added [α -³²P]dCTP to the various extracts, and assessed both the rate and extent of chromosomal DNA synthesis (Fig. 3a). We observed that total DNA synthesis in the XORC2-depleted extracts was severely diminished (10% or less of the level in the mock-depleted extracts) (Fig. 3b).

We examined whether the replication defect in the XORC2-depleted extracts could be rescued by the addition of purified XORC2 protein. However, recombinant XORC2 at concentrations up to 20 ng μl^{−1} was not effective in reversing this defect. Because budding yeast Orc2 associates with several other polypeptides^{1–9}, the inability of recombinant XORC2 to rescue the depleted extracts may be due to the immunodepletion procedure removing additional XORC2-associated proteins. To investigate this possibility, we subjected egg cytosol fractions to gel filtration in a Superdex 200 column (Fig. 3d). Approximately half of the XORC2 protein migrated with an apparent *M_r* > 440K, which suggests that it resides in a multiprotein complex.

To exclude the possibility that the XORC2-depleted extracts might be irreversibly inactivated, we added cytosol that had been precipitated with 32.5% ammonium sulphate to these extracts. This ammonium sulphate fraction elicited a significant restoration of DNA synthesis in XORC2-depleted extracts (Fig. 3c). After normalizing for the concentration of XORC2 protein in this fraction, we determined that replication could be restored to 85% of the level in the mock-depleted extract. In another approach, by mixing control and XORC2-depleted extracts we showed that the total amount of DNA replication in both extracts

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FIG. 1 a, Rescue of a fission-yeast mitotic-catastrophe strain (SP984, *mik1::ura4⁺ wee1-50 leu1-32 ade6-210 ura4-D18 h⁺*)¹⁴ by a *Xenopus* cDNA encoding XORC2. SP984 transformed with pAX-NMT containing the



b

Xl-Orc2	M S H E A H N G T K L F K V Q F T P D E E V L E H I T D K Q G V T P Q S - N T A A Q R I V A V K N I I K N D D L H V D E A T P E S W R E Q N Y V	71
Sc-Orc2	----- M L N G E D F V E H N D I L S S P A K S R N V T P K R V D P H G E R Q L R R I H S S K K N L L E R I S L V G N E R K N T S P D	63
Xl-Orc2	D V L G V D T E D T L Q N G P T G G G D V Y S F Y T I K R S N K M A Q L A T E M A R T P A K T V S F S V S D N P E E A V S P P A N K Q T E N K	143
Sc-Orc2	P A L K P K T P S K A P R K R G R P R K I Q E E L T D R I K K D E K D T I S S K K K R K L D K - - - D T S G N V N E S K T S N K Q V M E K	131
Xl-Orc2	T P S K G R K - - - - - N E F I S T T P H R L R K R L S A P S Q P S D S E S D Y S A S N S D - - - - - E E L E E - K S	191
Sc-Orc2	T G I K E K R E R E K I Q V A T T Y E D N V T P Q T D D N F V S N S P E P P E P A T P S K K S L T T N H D F T S P L K Q I I M N N K E Y K D	203
Xl-Orc2	S A A K T P S K V S Q T P G K T P S K K K K K - - - - - N T A S N L V E E Y F E A H S S S K V L T S D R T L Q R L Q T F K L D Q E T L R K L	256
Sc-Orc2	S T S P G K L T L S R N F T P T P V P K N K K L Y Q T S E T K S A S S F L D T F E G Y F D Q R K I V R T N - - A K S R H T M S M A P D V T R E E	273
Xl-Orc2	L D Q T P S A F A D E L H R I S K K - - - - - H E A L F Y K W M L Q L H L G F N I I L F G L G S K Q S L I B K F R T S L Q D S L - - - - -	316
Sc-Orc2	F S L V S N F F N E N F Q K R P R Q K L F E I Q K K M F P Q Y W F E L T Q G F S L L F Y G V G S K R N F L E E F A I D Y L S P K I A Y S Q L A Y	345
Xl-Orc2	- - - - - H I V I N E F F P S I T A K S I L N S I T E E A L G H P G A F R S - - - - - P L D Q M D F I L Q R F K D D P -	365
Sc-Orc2	E N E L Q Q N K P V N S I P C L I L N G Y N P S C N Y R D V F K E I T - D L L V P A E L T R S E T K Y W G N H V I L Q I Q K M I D F Y K N Q P L	416
Xl-Orc2	S L E L V L I H N L D S Q M L R G E K C Q Q V L G Q L A S T P N L H L L A S V D H I N A P L M W D Q S K Q S L Y N W L W Y E T T T F G S Y I E	437
Sc-Orc2	D I K L I L V V H N L D G P S I R K N T F Q T M L S F L S V I R Q I A I V A S T D H I Y A P L L W D N M K A Q N Y N F V F H D I S N F E P S T V	488
Xl-Orc2	E T S Y E N S L - L V K R S G A L A L S S L T W V L R S L T P N A R G I F R L L A E Y Q M A N K D N P S Y - - - - - T G L S F Q D F Y	498
Sc-Orc2	E S T F Q D V M K M G K S D T S S G A E G A K Y V I L Q S L T V N S K K M Y K L L I E T Q M Q N M G N L S A N T G P K R G T Q R T G V E L K L F N	560
Xl-Orc2	Q Q C R E A F L V N S D L T L R A Q L T E F R D H K L I R T K K G M D G V E Y L L I P I D L G T L T D F L E M E D K D T	558
Sc-Orc2	H L C A A D F I A S N E I A L N S M L R E F I E H K M A N I T K N N S G M E I I W V P Y T Y A E L E K L L K T V L N T L	620

XORC2 cDNA or no insert was grown for 6 days at 35.5°C. SP984 transformed with pAX-SV40 containing the wild-type *S. pombe wee1* gene was streaked out in parallel. b, Amino acid alignment of XORC2 with Orc2 from *S. cerevisiae*³⁻⁵.

METHODS. A *Xenopus* oocyte cDNA library was prepared in a *S. pombe* expression vector (pAX-NMT) as described²⁴, except that the plasmid contained the *nmt1* promoter²⁵. This shuttle vector contains the pBR322 origin of replication, the β -lactamase gene, the *S. cerevisiae LEU2* gene, and the *S. pombe ars1* element for propagation in *Escherichia coli* and *S. pombe*. Strain SP984 was transformed with the plasmid library by the spheroplast method using lipofectin^{26,27}. From 2.3×10^5 transformants, 60 colonies that grew at the restrictive temperature of 35.5°C on minimal medium (PM) lacking leucine and thiamine were obtained. The plasmid from one rescuer (pC337) was chosen for further study. pC337 rescued the SP984 strain upon retransformation in the absence, but not the presence, of thiamine. The nucleotide sequence of the 2.1-kb *Apal/XhoI* insert in pC337 was determined by standard methods (GenBank accession number U31984).

FIG. 2 Characterization of anti-XORC2 antibodies. a, Coomassie blue staining (lane 1; 2.5 μ g protein) and immunoblotting (lane 2; 20 ng protein) of purified, histidine-tagged XORC2 protein. b, An interphase extract from *Xenopus* eggs (lane 1; 50 μ g protein) and a lysate from XTC cells (lane 2; 30 μ g protein) were immunoblotted with anti-XORC2 antibodies. The autoradiogram in lane 2 was exposed substantially longer than that in lane 1. c, M-phase (lanes 1 and 3) and interphase egg extracts (lane 2) were immunoblotted with anti-XORC2 antibodies. d, Immunodepletion of XORC2 from interphase egg extracts. Supernatants from extracts that had been treated with either anti-XORC2 (lane 1) or control antibodies (lane 2) were immunoblotted with anti-XORC2 antibodies. Lane 3 depicts a portion of the immunoprecipitated XORC2 protein from the extract used in lane 1. In this experiment, 95% of the XORC2 protein was removed. e, DNA staining and phase-contrast microscopy of nuclei assembled around sperm chromatin in either a mock-depleted (top) or XORC2-depleted (bottom) extract. The width of each panel corresponds to 100 μ m.

METHODS. The XORC2-coding sequence was cloned into the baculoviral expression vector pVL-HIS^{24,28} after creation of an *NdeI* site at the initiation codon and elimination of an internal *NdeI* site. The recombinant XORC2 protein containing a six-histidine tag at its N-terminal end (His6-XORC2) was produced in Sf9 insect cells and purified as described^{24,28}. Typically, from a 100-ml culture of Sf9 cells, we obtained ~0.5 mg of His6-XORC2 that was greater than 90% pure, as judged by Coomassie blue staining. His6-XORC2 was used to generate polyclonal rabbit antibodies, which were affinity purified as described^{24,28}. Unactivated *Xenopus* egg extracts (M phase) or egg extracts activated by the addition of calcium (interphase) were prepared as described^{24,28}. For immunodepletion of XORC2, 100- μ l portions of extract (15 min postactivation) were treated twice for 45 min at 4°C with 20 μ l of protein A beads containing 15 μ g of either anti-XORC2 antibodies or a control rabbit IgG. After immunoblotting of the supernatants and pellets, the concentration of XORC2 was quantified by using a PhosphorImager (Molecular Dynamics). Nuclei in *Xenopus* extracts were stained with Hoechst 33258 and photographed using a Zeiss Axiophot microscope^{24,28}.

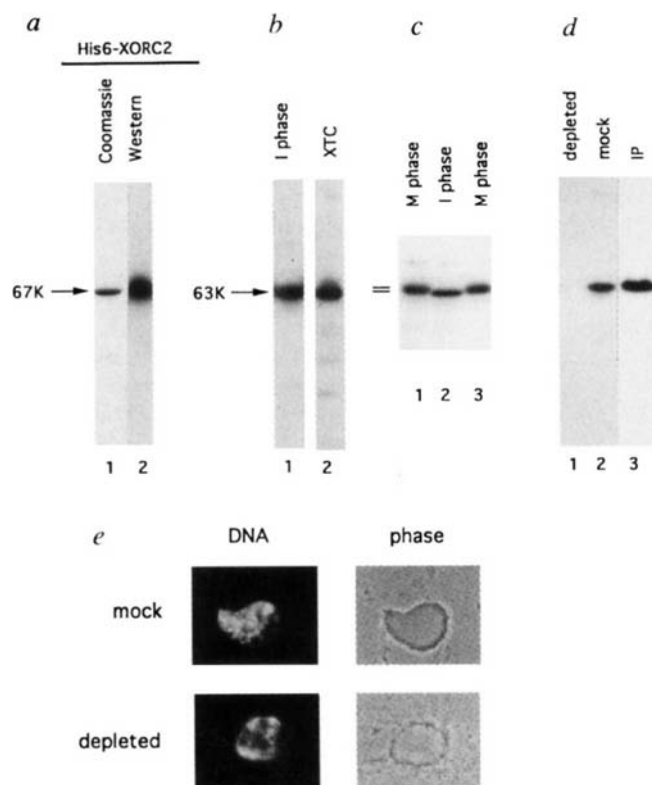


FIG. 3 Role of XORC2 in DNA replication in *Xenopus* egg extracts. **a**, Replication of sperm chromatin was determined in the XORC2-depleted and mock-depleted interphase extracts shown in Fig. 2d. **b**, The replication of sperm chromatin was quantified in three independent depletion experiments. Column 2 represents the experiment shown in **a**. The quantification represents the sum of pulse labellings with [α - 32 P]dCTP from 0–60 min, 60–90 min, 90–120 min, 120–150 min and 150–180 min. **c**, Fractionated egg cytosol restores replication to XORC2-depleted extracts. Chromosomal replication was measured by a continuous labelling with [α - 32 P]dCTP over a 90-min period in a control-depleted (lane 1) or a XORC2-depleted extract to which had been added buffer alone (lane 2), the 50–150 mM NaCl eluate of egg cytosol fractionated on a DE52 column (lane 3), or the 32.5% ammonium sulphate fraction from egg cytosol (lane 4). The XORC2 protein was depleted by greater than 99% (the limit of detection) in this experiment. **d**, Partially purified XORC2 was fractionated in a Superdex 200 column. **e**, Replication of single-stranded M13 DNA was measured over a 3-h period in control (column 1) and XORC2-depleted extracts (column 2). The average of three experiments is shown.

METHODS. Replication of sperm chromatin (1,000 nuclei per μ l) or M13 DNA (1 ng μ l $^{-1}$) was monitored by agarose gel electrophoresis following pulse labelling with [α - 32 P]dCTP as described previously^{18,23}. Quantification of 32 P incorporation into DNA was performed with a PhosphorImager. *Xenopus* egg cytosol was prepared by centrifugation of interphase extracts for 90 min at 55,000 r.p.m. with a Beckman TLS 55 rotor. For ammonium sulphate precipitation, the cytosol was diluted twofold with ELB²⁹, and mixed with a saturated ammonium sulphate solution to achieve a final concentration of 32.5%. The precipitated proteins were dissolved in XB²⁴ containing 1 mM dithiothreitol and dialysed against the same buffer. Before DEAE-cellulose (DE52) chromatography, cytosol was diluted threefold with buffer D (50 mM Tris-HCl, pH 8.0). The column was washed with buffer D containing 50 mM NaCl and eluted with buffer D containing 150 mM NaCl. The XORC2 protein in this 50–150 mM NaCl eluate was substantially diluted, and therefore this eluate restored replication only weakly to the XORC2-depleted extract shown in Fig. 3c (lane 3). Before gel filtration of XORC2, the 50–150 mM NaCl fraction from the DE52 column was concentrated on a heparin-Affigel column (BioRad) equilibrated in buffer D. The 50–200 mM NaCl eluate from the heparin column was chromatographed on a Superdex 200 PC column using a SMART system (Pharmacia). The XORC2-containing fractions were determined by immunoblotting. The column was calibrated with blue dextran (V_0 , void volume), thyroglobulin (M , 670K), ferritin (440K), aldolase (158K), bovine serum albumin (68K), and ovalbumin (43K).

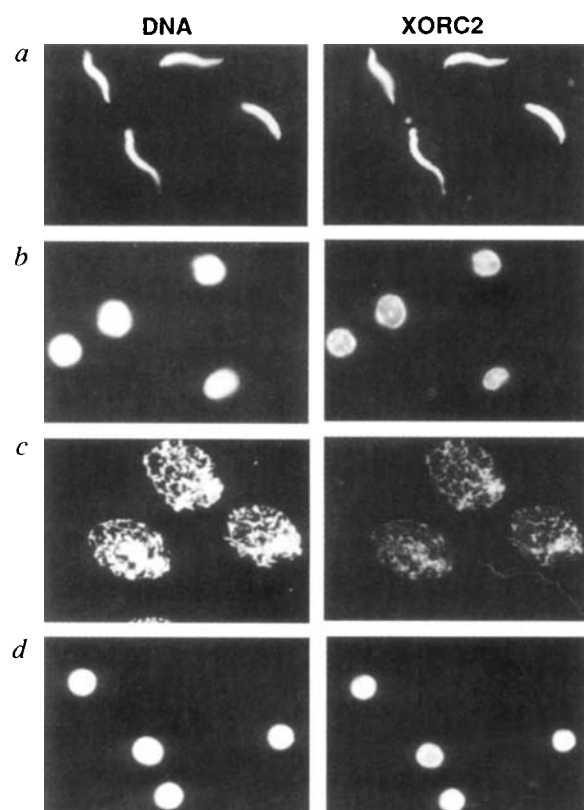
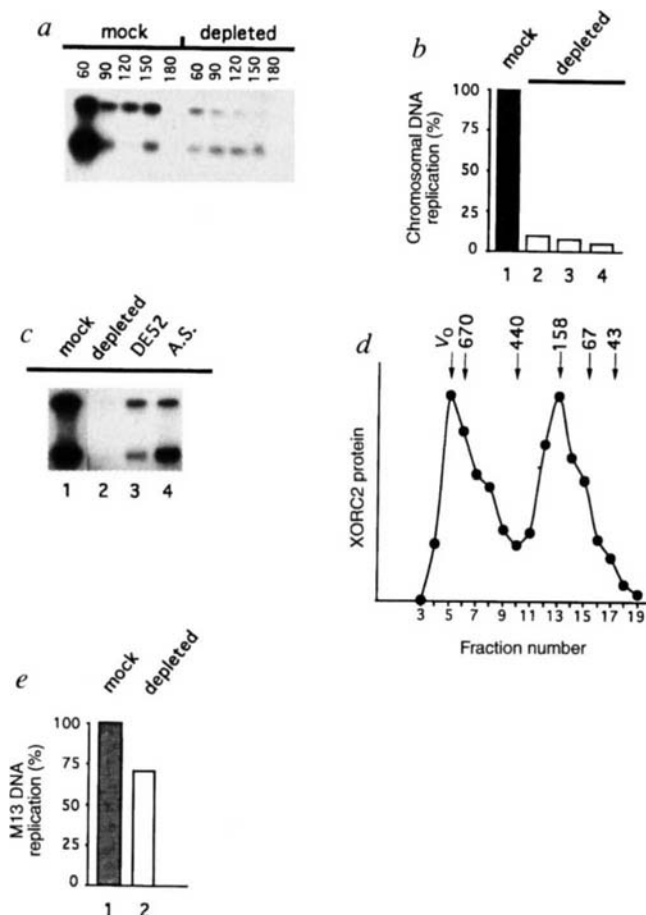


FIG. 4 Detection of XORC2 by indirect immunofluorescence on chromatin in *Xenopus* egg extracts. Sperm chromatin was incubated in interphase extracts for 15 min (**a**), 30 min (**b**) or 60 min (**c**). In **d**, the chromatin was incubated for 15 min in an extract containing 5 mM 6-DMAP. Subsequently, the chromatin was stained with Hoechst 33258 (left) and subjected to indirect immunofluorescence with anti-XORC2 antibodies (right). The width of each panel corresponds to 125 μ m.

METHODS. Extracts that were supplemented with demembrated sperm chromatin (1000 nuclei per μ l) were fixed by diluting twofold in ELB as described²⁹. The fixed extracts were layered on a sucrose cushion, and the nuclei were pelleted onto coverslips^{29,30}. Indirect immunofluorescence was performed by standard methods^{29,30} with fluorescein-conjugated goat anti-rabbit IgG (Cappel) in the secondary step. The samples were mounted in a glycerol solution containing 10 μ g ml $^{-1}$ Hoechst 33258. No chromatin staining was observed with a control rabbit IgG in the primary step (not shown). The samples were viewed with a $\times 63$ oil-immersion objective and photographed at identical magnification and exposure times with a Zeiss Axiophot microscope.

was additive (not shown), indicating that there is no inhibitor of replication in XORC2-depleted extracts. To characterize further the replication defect in the XORC2-depleted extracts, we examined whether these extracts could perform elongation on a single-stranded M13 DNA template. This reaction does not require that the DNA be incorporated into a nucleus, and is thought to reflect the ability of the extracts to perform an elongation mode of DNA replication¹⁷⁻¹⁹. XORC2-depleted extracts were found to replicate M13 DNA efficiently (71% of control extracts) (Fig. 3e), suggesting that elongation is not strongly compromised by the removal of XORC2 and any associated proteins. Taken together, these experiments indicate that immunodepletion of XORC2 from *Xenopus* egg extracts results in a complete, but reversible, inhibition of an early step in chromosomal replication.

To examine the properties of XORC2 in greater detail, we used indirect immunofluorescence to determine its subcellular localization at various stages of DNA synthesis in *Xenopus* egg extracts (Fig. 4). We observed that XORC2 associated uniformly and rapidly (within 15 min) with chromatin that had been added to activated egg extracts (Fig. 4a), by which time, nuclear envelope assembly is not yet complete. Furthermore, as determined by both radiolabelling with [α -³²P]dCTP and indirect immunofluorescence with biotinylated dUTP, there is no detectable DNA synthesis within this early 15-min period (not shown). At later times (30 and 60 min), when the extracts are actively engaged in DNA synthesis, XORC2 could also be detected on chromatin throughout the assembled nuclei (Fig. 4b,c). In *Xenopus* egg extracts, the kinase inhibitor 6-dimethylaminopurine (6-DMAP) abolishes replication and prevents the association of the XMCM3 protein, a proposed component of licensing factor, with chromatin²⁰⁻²². Although 6-DMAP inhibited replication by more than 95% (not shown), it had no effect on the ability of XORC2 to associate with the sperm chromatin (Fig. 4d). This observation implies that XORC2 can bind to chromatin independently of licensing factor components such as XMCM3, and suggests that XORC2 acts upstream of XMCM3 in a sequence of events leading to replication. Collectively, these immunofluorescence observations indicate that the association of XORC2 with chromatin occurs in a manner consistent with a role in the initiation of DNA synthesis.

Replication of exogenous DNA in early *Xenopus* embryos occurs in a sequence-independent manner (reviewed in ref. 10). Our observation that replication in *Xenopus* egg extracts proceeds by a XORC2-dependent mechanism raises the possibility that chromosomal DNA replication in this system involves the use of discrete origins. It will be valuable to ascertain whether XORC2 and its putative associated proteins bind preferentially to defined chromosomal sequences, and whether this preference might vary during development. As well as the initiation of DNA synthesis, many aspects of how S phase is integrated properly into the cell cycle can be studied in *Xenopus* extracts. These processes include the block to re-replication that is controlled by licensing factor²⁰⁻²², and the coupling of mitosis to the completion of S phase through checkpoint mechanisms²³. The capability to analyse key replication proteins such as XORC2 at a biochemical level in the context of these regulatory processes may yield new insights into cell-cycle control. □

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Interaction of Cdc2 and Cdc18 with a fission yeast ORC2-like protein

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In fission yeast, Cdc2 kinase has both positive and negative roles in regulating DNA replication, being first necessary for the transition from G₁ to S phase and later required to prevent the re-initiation of DNA replication during G₂¹⁻³. We report here that Cdc2 interacts with Orp2, a protein similar to the Orc2 replication factor subunit of *Saccharomyces cerevisiae* origin recognition complex (ORC). ORC binds chromosomal origins and is essential for chromosomal replication initiation⁴⁻⁶. Fission yeast Orp2 is required for DNA replication and interacts with the rate-limiting replication activator Cdc18. Cells lacking Orp2 undergo aberrant mitosis, indicating that Orp2 is involved in generating a checkpoint signal. These findings suggest that ORC functions are conserved among eukaryotes and provide evidence that Cdc2 controls DNA replication initiation by acting directly at chromosomal origins.

Potential targets of Cdc2 kinase in the fission yeast *Schizosaccharomyces pombe* were identified using a two-hybrid screen in the budding yeast *S. cerevisiae*⁷. A screen of 2 × 10⁷ fission yeast cDNA clones yielded 112 positives representing seven genes. One, *orp2*⁺ (ORC2 related in *pombe*), encodes a protein which is 22% identical to the Orc2 subunit of *S. cerevisiae* ORC (Fig. 1). ORC specifically binds to a conserved DNA sequence element of replication origins and is required for replication initiation *in vivo*^{4-6,8-12}. ORC appears to remain bound to origins throughout the cell cycle and may serve as a platform for assembly of replication-competent complexes during G₁^{13,14}. Consensus cyclin-dependent kinase phosphorylation sites in the Orc2 subunit led to speculation that Orc2 might be regulated by Cdc28, the budding yeast Cdc2 homologue⁴. Similar sequences in Orp2 and the identification of Orp2 by Cdc2 binding further support this idea (Fig. 1c).

To determine whether Cdc2 and Orp2 interact in *S. pombe*, functional GST-Orp2 fusion protein and a GST control were expressed in *S. pombe* and isolated by binding to GSH-Sepharose. Cdc2 was detected in association with the GST-Orp2 fusion but not with GST (Fig. 2a). Having verified the Cdc2 interaction, we used genetic approaches to test whether Orp2 functions in fission yeast replication.

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The function of *orp2*⁺ was investigated by germinating spores from an *orp2*⁺/*orp2::ura4*⁺ diploid in media lacking uracil (Fig. 2b–d). The *Δorp2* cells progressed normally through one cycle of DNA replication and cell division. After this, *Δorp2* cells accumulated as long cells with about 2C DNA content, indicating that the second mitosis was delayed. Thus *Δorp2* cells replicated their DNA roughly twice, possibly using inherited Orp2 protein. After 14 h, *Δorp2* cells continued to grow, but cell number increased slowly, cells with less than 2C DNA content appeared, and aberrant mitoses became apparent by microscopic analysis. In many cells, DNA masses separated unequally and incompletely, remaining connected by threads of chromatin (Fig. 2d). Septum formation and cytokinesis in such cells was visible and may account for the broad distribution of cells having less than 2C DNA content.

We suspected that the *Δorp2* phenotype resulted from a defect in DNA replication combined with a failure of the checkpoint control that prevents the onset of mitosis before the completion of replication¹⁵. We therefore investigated the consequences of the *Δorp2* mutation in a situation that was not complicated by phenotypes arising from an abnormal mitosis. Cells lacking *cdc13*⁺, a cyclin-B gene, undergo multiple DNA replication cycles without mitosis, resulting in cells with huge nuclei³. Cells containing a *Δcdc13* allele (*cdc13::his7*⁺) were compared with double-mutant *Δorp2 Δcdc13* cells in a selective spore germination experiment. Spores of both genotypes germinated and elongated without nuclear division or septum formation. The *Δcdc13*

cells re-replicated DNA, accumulating up to 32C DNA content by 16 h and forming gigantic nuclei (Fig. 3). In contrast, there was no indication of multiple rounds of re-replication in *Δorp2 Δcdc13* cells, and even after 36 h no gigantic nuclei were observed (Fig. 3 and data not shown). The *orp2 Δcdc13* cells accumulated roughly 4C DNA content, indicating that they replicated DNA roughly twice as seen in the *Δorp2* mutant. These data demonstrate that Orp2 is essential for replication, although they do not exclude an additional role for Orp2 in mitosis.

Orp2 overproduction (OP-Orp2) delayed mitosis, generating moderately elongated cells having a 2C DNA content (data not shown). The delay was independent of Orp2's essential function because a non-complementing allele of Orp2 had a similar effect. The delay phenotype was synergistic with *cdc2-33* and *cdc13-117* mutations, indicating that it resulted from inhibition of Cdc2 activity. It was possible that OP-Orp2 delayed mitosis by interfering with DNA replication and thereby invoking a checkpoint signal. This possibility was investigated by overexpressing Orp2 in a *rad1-1* mutant. Rad1 is required for checkpoint control, hence *rad1-1* mutants are very sensitive to the DNA replication inhibitor hydroxyurea (HU)¹⁶. We found that OP-Orp2 rescued the HU sensitivity of a *rad1-1* mutant (Fig. 4a). Microscopic analysis showed that OP-Orp2 delayed mitosis independent of the *rad1-1* mutation, and therefore OP-Orp2 prevented the aberrant nuclear divisions resulting from HU treatment of the *rad1-1* mutant (Fig. 4b). Thus OP-Orp2 delays mitosis by a Rad1-independent checkpoint pathway or by inhibiting Cdc2 directly,

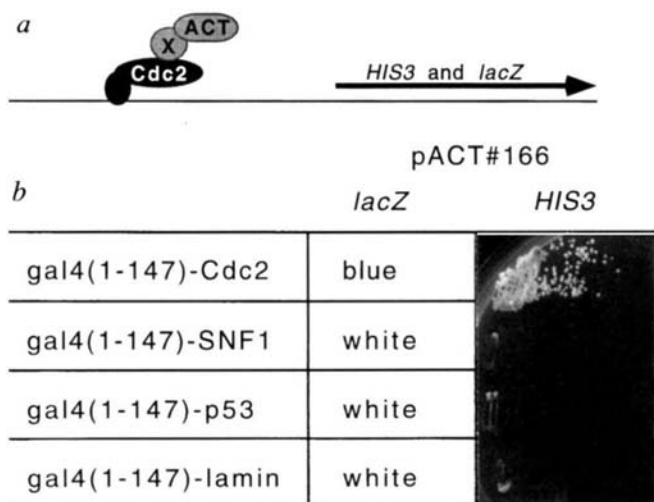


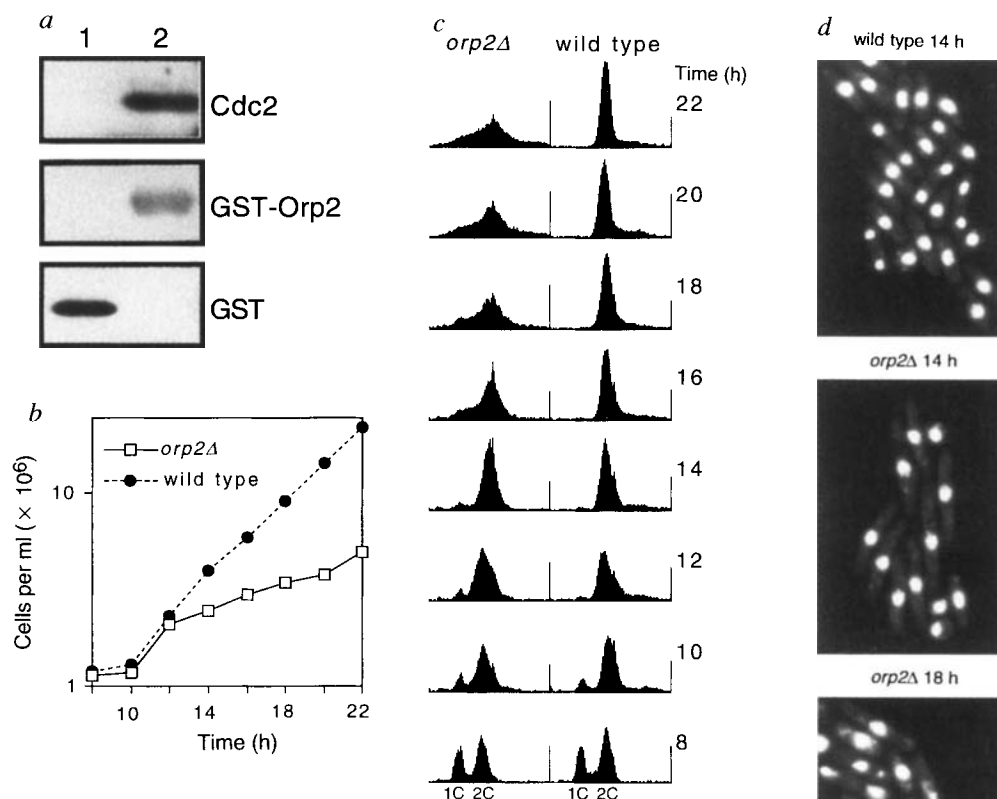
FIG. 1 a, A two-hybrid screen was used to isolate Cdc2 interacting factors. Protein interactions that bring the activator, *gal4*(region II), to the DNA-bound *gal4*(1-147)-Cdc2 protein activate transcription of *HIS3* and *lacZ* constructs. A total of 112 Cdc2 specific isolates were analysed and found to represent seven genes including *orp2*⁺ and *suc1*⁺. **b**, The *gal4*(region II)-Orp2 construct expressed from isolate pACT#166 activates transcription in combination with *gal4*(1-147)-Cdc2 but not with *Gal4*(1-147) fused to SNF1, p53 or Lamin. *S. cerevisiae* strain Y190 was cotransformed with pACT#166 and pAS vectors expressing the *gal4*(1-147) fusions as indicated. *HIS3* expression promotes growth on 30 mM 3-amino-triazole (right column) and *lacZ* expression turns X-gal-incubated colonies blue (middle column). **c**, Alignment of *S. pombe* (S.p.) Orp2 with *S. cerevisiae* (S.c.) Orc2. Identities are shaded. Cdc2 phosphorylation motifs (S/T-P) are boxed; those most closely matching Cdc2/Cdc28 phosphorylation sites are outlined in bold.

METHODS. A library of *S. pombe* cDNAs expressed 3' of *gal4*(region II) in pACT was transformed into *S. cerevisiae* strain Y190 containing *gal4*(1-147)-Cdc2. *HIS3*-expressing colonies were selected from 2×10^7 transformants and screened for high *lacZ* expression essentially as described²⁴. Mapping by hybridization of pACT#166 to cosmid and P1 clones localized *orp2*⁺ on chromosome II between *top1*⁺ and *cdc10*⁺ (refs 25, 26). P1 clone 7B10p was digested with *HindIII* and the 3 kb pACT#166 hybridizing band was ligated into *HindIII*-digested pBluescriptSK to make pER17 and into

HindIII-digested pSAB1 to make the *S. pombe* vector pER27. pER27 complements the *orp2::ura4*⁺ mutant described in Fig. 2. The *orp2*⁺ nucleotide sequence was determined by dideoxynucleotide sequencing of both strands of isolate pACT#166 (amino acids 38–490 of Orp2) and the flanking regions of pER17 (GenBank accession #U38472); the predicted amino-acid sequence of the single open reading frame which lacks introns is shown.

FIG. 2 *a*, Cdc2 and Orp2 associate *in vivo*. Lysates from *S. pombe* cells expressing GST (lane 1) or GST-Orp2 (lane 2) were incubated with GSH-Sepharose and extensively washed; the bound proteins were separated by SDS-PAGE and analysed by immunoblotting with antibodies specific for GST (lower two panels) or Cdc2 (top panel). Cdc2 was specifically associated with GST-Orp2. GST-Orp2 complements the *orp2::ura4⁺* allele (data not shown). *b*, Spores from congenic diploids having either *ura4⁺* (wild-type) or *orp2::ura4⁺* were selectively germinated to grow only the *Ura⁺* cells. At 0 h, spores were inoculated in EMM2 supplemented with leucine, adenine and uracil at 30 °C. Germinated cell number (starting concentration of $1 \times 10^6 \text{ ml}^{-1}$) is shown on a $\log_{10}$ scale as a function of time after inoculation. Germination was visible at 8 h, division began after 10 h, and cell number for Δorp2 and wild-type doubled by 12 h. Cells were maintained in logarithmic growth by additions of fresh media. *c*, Δorp2 cells arrest with DNA contents of $\sim 2C$ and less. DNA content was measured by fluorescence-activated cell sorting (FACS)²⁷. 1C and 2C positions were determined using a *cdc10-129* mutant strain; spores were gated out on the basis of forward scatter. *d*, Δorp2 cells undergo a cell-cycle arrest followed by an abnormal mitosis in which nuclei are divided unequally by the division septum. Nuclei were stained with $1 \mu\text{g ml}^{-1}$ DAPI.

METHODS. GST and GST-Orp2 expression was induced by growth at 30 °C in EMM2 lacking thiamine for 15 h. Cells were disrupted with glass beads in buffer L (50 mM Tris pH 8.0, 150 mM NaCl, 1% Nonidet-P40, 5 mM EDTA, 10% glycerol, 1 mM PMSF, $5 \mu\text{g ml}^{-1}$ pepstatin, $5 \mu\text{g ml}^{-1}$ leupeptin and $5 \mu\text{g ml}^{-1}$ aprotinin), and lysates were centrifuged at 16,000g for 5 min at 4 °C. GSH-Sepharose (20 μl ; Pharmacia) was added to 400 μl of supernatant ($15\text{--}20 \text{ mg ml}^{-1}$ protein concentration), incubated at 4 °C for 2 h, and washed three times in buffer L. Immunoblot detection was by ECL (Amersham). The *nmt1* promoter drives expression of GST and GST-Orp2 in pJL205 (GST inserted between *NdeI* and *BamHI* of pREP1²⁸) and pJL211 (the 1.8 kb *orp2⁺* *BamHI* fragment of pJL201 ligated into *BamHI*-digested pJL205). The *orp2⁺* coding region was amplified using oJB15 (5'-AAAAG-



GATCC ATTGGCCAAT GCTAATAAAC GAGCAGG-TAC-3') and oJB14 (5'-AAAAGGATCC ATACAATTGC ACCAATGTGC-3'), digested with *Bam*HI, and ligated into *Bam*HI-digested pBlue-scriptSK creating pJL201. Construction of JLP46: the 1.4 kb *Xho*I fragment of pACT#166 was ligated into *Xho*I-digested pJL164 (pBlue-scriptSK deleted from *Not*I to *Xho*I) creating pER1; the coding region of *orp2⁺* between *Bst*B1 and *Xba*I was replaced with *ura4⁺* creating the *orp2::ura4⁺* allele in pER6; pER6 linearized with *Xho*I-EcoRI was transformed into a diploid, stable *Ura⁺* colonies were screened by southern blot and JLP46 and JLP47 were isolated (*h⁺/h⁻ orp2::ura4⁺/orp2⁺ ura4-D18/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1-32 his7-366/his7-366*). The congenic wild-type strains are JLP51 and JLP52 (*h⁺/h⁻ orp2⁺/orp2⁺ ura4⁺/ura4-D18 ade6-M210/ade6-M216 leu1-32/leu1-32 his7-366/his7-366*). JLP46 and JLP51 are shown; identical results were obtained with JLP47 and JLP52.

perhaps by binding to Cdc2 and restricting its access to other substrates.

Origin recognition factors have remained elusive in organisms other than budding yeast, in part because the *cis*-acting determinants of replication origins have been difficult to characterize. Replication origin sequence requirements differ between *S. cerevisiae* and *S. pombe*^{17,18}, thus it was uncertain whether replication initiator proteins should be conserved. The demonstration that Orp2 is required for DNA replication suggests that Orc homologues are relevant to understanding the conserved properties of origin function. However, note that *orp2⁺* expression failed to rescue the DNA replication defect of *S. cerevisiae* *orc2-1*, *orc2-2* or *orc2-3* mutants (data not shown). This may reflect a role for Orc2 in DNA sequence recognition, a supposition consistent with studies showing that Orc2 is one of three ORC subunits juxtaposed to DNA⁸.

A role of Orp2 in regulating DNA replication was further supported by the discovery that it interacts with Cdc18, a rate-limiting activator of DNA replication^{19,20}. This interaction was first detected as a twofold transcriptional activation in a yeast two-hybrid assay (data not shown), and was confirmed by finding that Cdc18 coprecipitated with GST-Orp2 in fission yeast lysates

(Fig. 4c). Genetic and biochemical evidence suggests that *S. cerevisiae* ORC interacts similarly with Cdc6, a Cdc18-like protein in budding yeast^{10,12,21-23}. Our data suggest that Orp2/Cdc18 interaction is a conserved *in vivo* association and may be a key aspect of Cdc18 function.

Identifying the interaction between Cdc2 and Orp2 suggests that Cdc2 kinase has a very direct role in regulating DNA replication. Cdc2 may act at replication origins, possibly by phosphorylating an initiator-ORC complex. It is striking that multiple Cdc2 phosphorylation site sequences are present in the amino termini of both the putative homologue pairs Orp2/Orc2 and Cdc18/Cdc6 (Fig. 1 and ref. 19). It will be interesting to investigate whether Cdc2 phosphorylation of these components prevents re-replication by restricting *de novo* Orp2 and Cdc18 associations to G₁ when Cdc2 activity is lowest. Current studies are focusing on whether Orp2 function is regulated by Cdc2 phosphorylation. □

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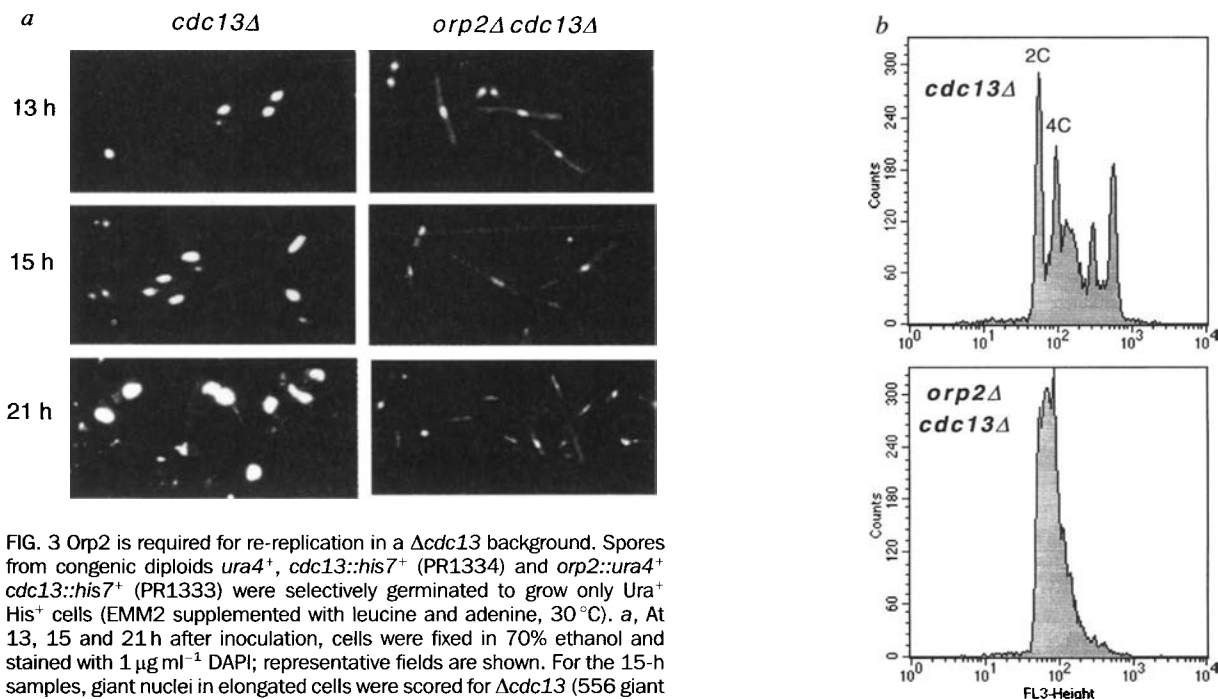


FIG. 3 Orp2 is required for re-replication in a $\Delta cdc13$ background. Spores from congenic diploids *ura4⁺, cdc13::his7⁺* (PR1334) and *orp2::ura4⁺ cdc13::his7⁺* (PR1333) were selectively germinated to grow only *Ura⁺ His⁺* cells (EMM2 supplemented with leucine and adenine, 30 °C). a, At 13, 15 and 21 h after inoculation, cells were fixed in 70% ethanol and stained with 1 $\mu\text{g ml}^{-1}$ DAPI; representative fields are shown. For the 15-h samples, giant nuclei in elongated cells were scored for $\Delta cdc13$ (556 giant nuclei per 1,000 cells) and for $\Delta cdc13 \Delta orp2$ (0 giant nuclei per 1,000 cells). b, DNA content of cells 16 h after inoculation was measured by FACS²⁷. The 4C position was determined using a diploid; spores and small cells were gated out based on forward scatter, and results are plotted on a log scale.

METHODS. The entire *cdc13⁺* coding region was replaced with *his7⁺* and transformed into diploids JLP46 and JLP51 to delete *cdc13⁺* (O. Mondesert and P. R., manuscript in preparation).

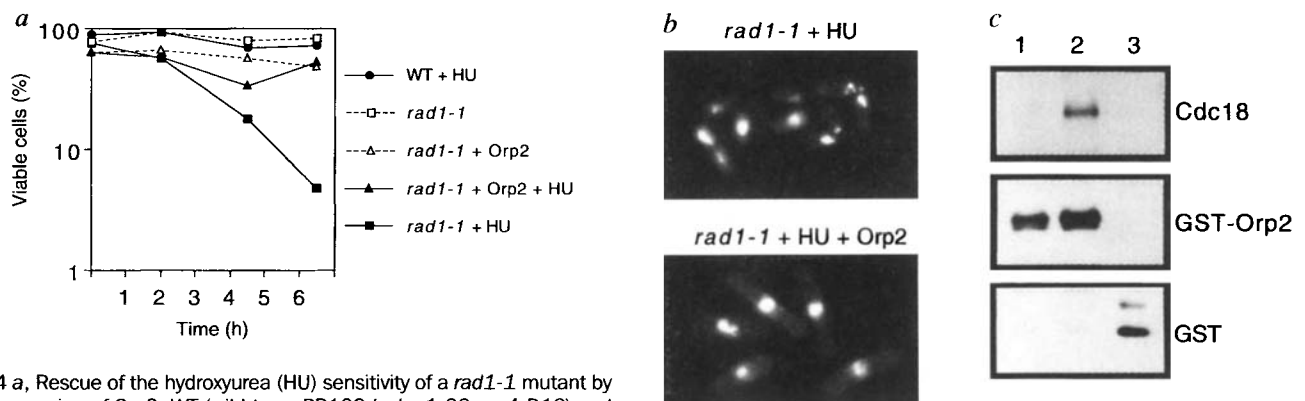


FIG. 4 a, Rescue of the hydroxyurea (HU) sensitivity of a *rad1-1* mutant by overexpression of Orp2. WT (wild-type, PR109 *h-leu1-32 ura4-D18*) and *rad1-1* (*h+rad1-1 leu1-32 ura4-D18 ade-*) cells were transformed with a vector control, pREP1²⁸, or with Orp2 expression plasmid pJL207. Orp2 was induced for 13 h in EMM lacking thiamine, cultures were divided and half received 10 mM HU at time 0 (+HU). Cell number was monitored and duplicate dilutions were plated on YES to determine viability. For clarity, the following strains which maintained high viability are not shown: WT, WT + Orp2, WT + Orp2 + HU. b, *rad1-1* mutant cells transformed with a vector control or with the Orp2 expression plasmid were treated as in a. Cells were fixed 6 h after HU addition, stained with DAPI, and photographed. c, Binding of Cdc18 to GST-Orp2 in fission yeast. *S. pombe* lysates derived from cells expressing GST-Orp2 (lane 1), GST-Orp2 and Cdc18-HA (lane 2), or GST

and Cdc18-HA (lane 3) were incubated with GSH-Sepharose; the bound proteins were separated by SDS-PAGE and analysed by immunoblotting with antibodies specific for GST (lower two panels) or HA (top panel). METHODS. The Orp2 coding 1.8 kb *Msc1-BamHI* fragment from pJL201 was ligated into *Msc1-BamHI*-digested pREP3²⁸ to create pJL207. Cdc18-HA was expressed from the *nmt1* promoter of pREP4-Cdc18-HA, a *ura4⁺* vector in which *cdc18⁺* with three copies of the HA epitope at the C terminus was inserted between *Msc1* and *BamHI* of pREP4²⁸. This construct complements *cdc18-K9*.

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Calcium oscillations in mammalian eggs triggered by a soluble sperm protein

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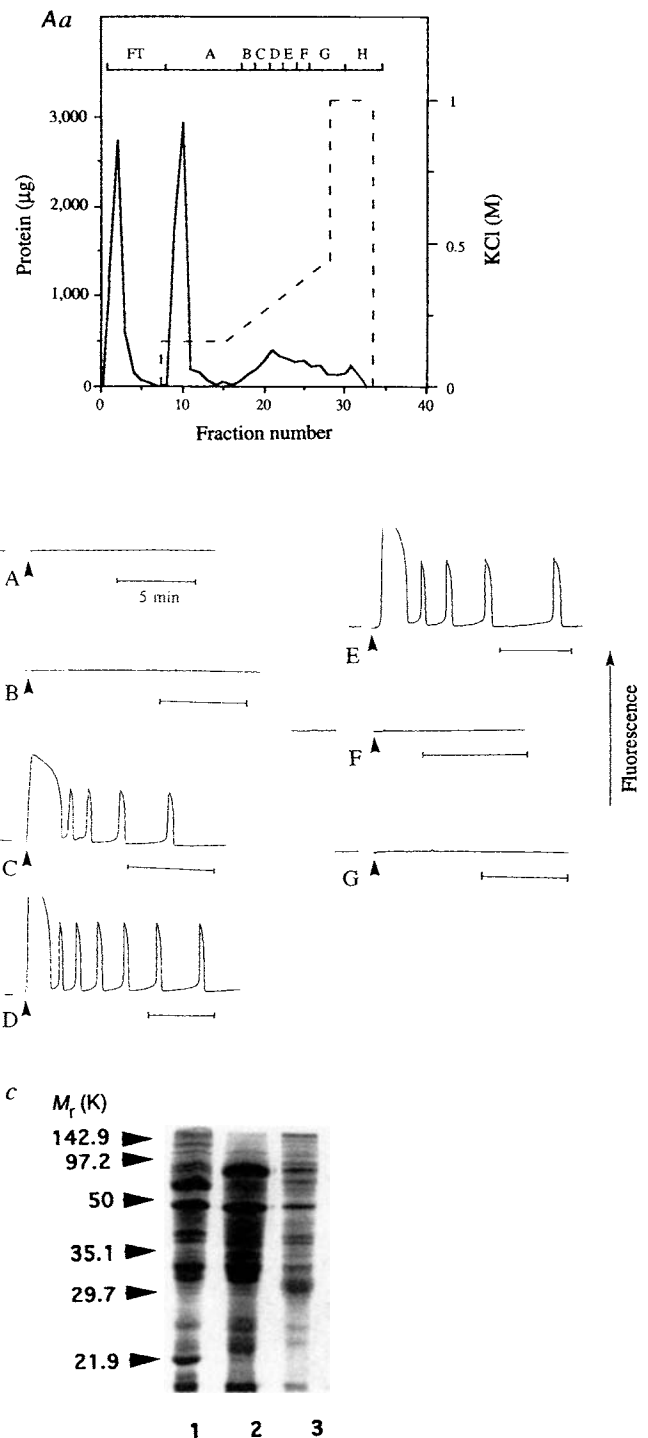
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At fertilization in mammals, the sperm induces a characteristic series of Ca^{2+} oscillations in the egg which serve as the essential trigger for egg activation and early development of the embryo¹⁻³. It is not known how the sperm initiates this fundamental process, however⁴⁻⁶, nor has any pathway linking sperm-egg membrane-receptor binding with intracellular Ca^{2+} release been demonstrated⁷⁻¹¹. Microinjection of sperm extracts into mammalian eggs elicits Ca^{2+} oscillations identical to those occurring at fertilization¹²⁻¹⁴, which suggests that sperm may introduce a Ca^{2+} oscillation-inducing factor into the egg on gamete membrane fusion^{12,15-18}. Here we identify a soluble sperm protein that exhibits Ca^{2+} oscillation-inducing ('oscillogen') activity in eggs. Sperm oscillogen exists as an oligomer with a subunit of M_r 33K and a specific intracellular localization at the equatorial segment of the sperm head. Cloning of the 33K oscillogen complementary DNA indicates similarity with a hexose phosphate isomerase found in prokaryotes. This sperm-derived oscillogen, termed oscillin, may represent the physiological trigger for development in mammals.

Sperm extracts that can produce Ca^{2+} oscillations indistinguishable from those observed in eggs during *in vitro* fertilization^{12,13} comprise a complex mixture of proteins (Fig. 1Aa). Soluble extracts from hamster sperm were separated using three successive chromatography columns, and the resulting fractions were screened for Ca^{2+} oscillation-inducing (oscillogen) activity (Fig. 1). Cytoplasmic free Ca^{2+} concentration was monitored in individual mouse eggs loaded with the Ca^{2+} -sensitive fluorescent dye, fluo-3, before and after microinjection with specific fractions eluted from each column. We used hamster sperm extracts because they possessed higher specific Ca^{2+} oscillogen activity than extracts from rat and boar, and mouse eggs because they were more sensitive than those of hamster in microinjection assays (data not shown). The specific fractions possessing Ca^{2+} oscillogen activity that eluted from sequential Cibachron Blue dye affinity and Mono Q ion-exchange columns (Fig. 1Aa, b) were loaded onto a hydroxyapatite column and proteins eluted with a linear phosphate gradient (Fig. 1Ba). After microinjection into mouse eggs, oscillogen activity was consistently observed only in samples C and D (fractions 15–18), corresponding to an elution position at 18–24 mM phosphate (Fig. 1Bb). Gel analysis of active fractions at each chromatographic step indicated the presence of a protein of relative molecular mass 33,000 (M_r 33K) that was also observed to comigrate specifically with the oscillogen activity eluted from the hydroxyapatite column (Fig. 1Bc, lanes 4 and 5). No other protein exhibited specific comigration with oscillogen activity; a 29K protein also found in the active fractions was present in much greater abundance in the earlier fractions (Fig. 1Bc, lanes 2 and 3), but these never triggered Ca^{2+} oscillations. This consistent comigration of the 33K protein with oscillogen activity suggests that it is the principal, if not sole, component of the sperm oscillogen. Although previous studies have indicated that G-protein activa-

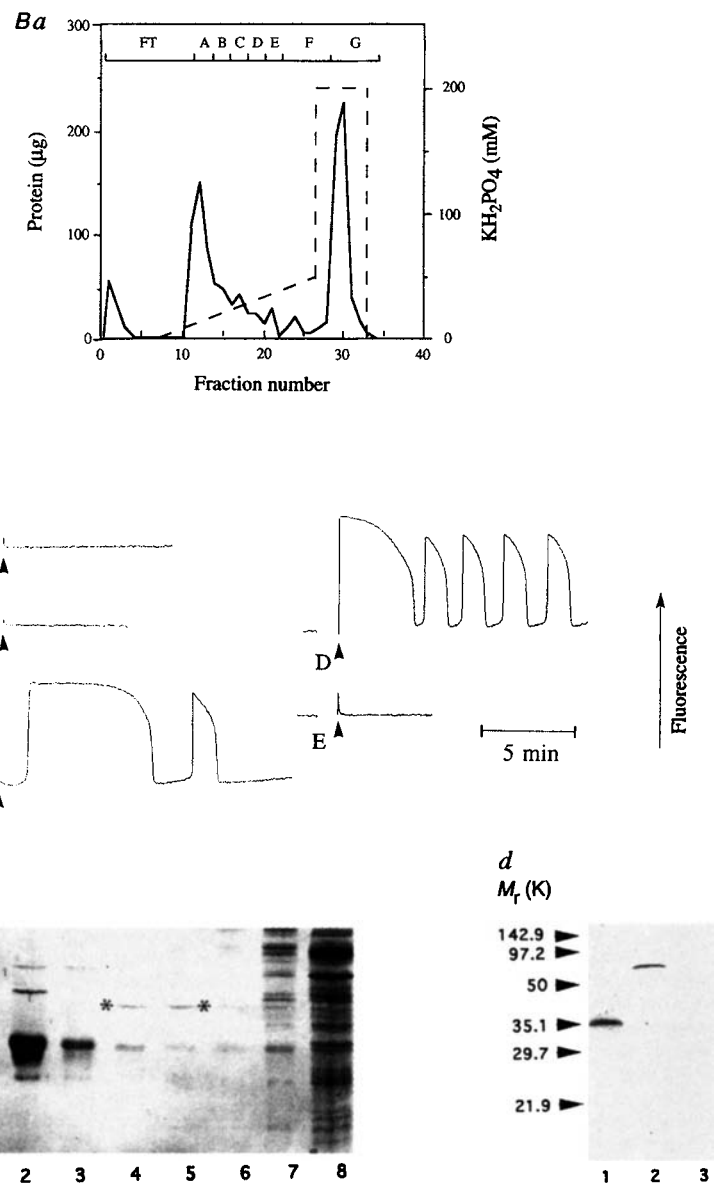


C	Total protein (mg)	Cumulative purification
whole sperm homogenate	1120	0
cytosolic extract	379	3
Cibachron-Blue eluate	17	66
Mono-Q FPLC eluate	1.13	991
Econo-HTP eluate	0.15	7467

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FIG 1 Chromatographic resolution of Ca^{2+} oscillogen activity with a soluble 33K hamster-sperm protein. **Aa**, Protein profile of KCl gradient elution of sperm extracts on Mono Q column. Fractions were pooled into samples FT (flowthrough) and A–H as indicated in inset. **b**, Fluo-3 fluorescence after microinjection into mouse eggs of samples A–G from Mono Q column. Injections were made at arrow; scale bars, 5 min. All samples except C–E were devoid of Ca^{2+} oscillogen activity (including FT and H). **c**, SDS gel of sperm crude cytosolic proteins (lane 1), Cibachron Blue column eluate (lane 2), and Mono Q column pooled active samples C–E (lane 3). **Ba**, Protein elution profile of sperm proteins on hydroxyapatite column chromatography. Fractions were pooled into samples FT and A–G as indicated in inset. **b**, Fluo-3 fluorescence after microinjection into mouse eggs of samples A–E from hydroxyapatite column. Injections were made at arrow; scale bars, 5 min. All samples except C and D were devoid of Ca^{2+} oscillogen activity. **c**, SDS gel analysis of hydroxyapatite column flowthrough (lane 1) and samples A–G (lanes 2–8). Asterisks denote position of the 33K protein present specifically in lanes 4 and 5 (samples C and D). **d**, Immunoblot analysis of soluble extracts from hamster testes (lane 1), sperm (lane 2) and hydroxyapatite column pooled fractions C and D (lane 3) probed with a panel of anti-G-protein-specific antibodies. **C**, Purification table summarizing the protein recovery at various steps in the oscillogen isolation procedure using motile sperm from cauda of 25 Syrian golden hamsters.

METHODS. Cytosolic extracts from motile hamster sperm, prepared by Cibachron Blue F3GA column chromatography as described previously¹³, were diluted into buffer A (20 mM HEPES, 1 mM EDTA, 200 μM PMSF, pH 7.5), and loaded onto a 1-ml Mono Q anion-exchange column using a Pharmacia LCC-500 FPLC system. After a 20-ml wash with buffer A and 10 ml of buffer A/150 mM KCl, proteins were eluted with a 30-ml linear gradient from 150 to 450 mM KCl, and finally with 10 ml of buffer A/1M KCl. Active samples were loaded onto a 1-ml hydroxyapatite column (Econo-HTP, Biorad), washed with 10-ml buffer A/100 mM KCl (minus EDTA), and eluted with a 20-ml linear gradient from 0 to 50 mM phosphate, and a final 200-mM phosphate step. Fractions were concentrated (Centricon-30) before assaying for Ca^{2+} oscillogen activity. The entire procedure from sperm homogenization to microinjection assay was completed within 28 h. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce). SDS-PAGE on 17.5% gels was performed as described previously²⁵ and stained with Coomassie Brilliant Blue R-250. Determination of apparent M_r was performed on linear 10–20% SDS-gradient gels, where the 33K sperm protein in hydroxyapatite column samples C and D migrated just below the 35K marker protein (Biorad). Zona free metaphase II mouse eggs were collected, prepared and maintained in M2 medium, pressure



injected (1–5% of egg volume) and intracellular Ca^{2+} measured with fluo-3 as described previously¹³. For immunoblot analysis, proteins were electrophoretically transferred onto Immobilon (Millipore), and incubated with the G-protein subunit antibody set (Calbiochem; anti- $\text{G}_\alpha 1$, anti- $\text{G}_\alpha 2$, anti- $\text{G}_\alpha 3$, anti- G_β , anti- G_γ , and anti-G-protein β -subunit, each at 1:1,000 dilution) and developed with horseradish peroxidase-conjugated anti-rabbit immunoglobulins (Dako) and 3,3'-diaminobenzidine/ H_2O_2 .

tion can lead to Ca^{2+} oscillations⁸, the 33K protein appears not to be one of the previously characterized G proteins, as it was not recognized by a panel of antibodies specific for the mammalian G proteins $\text{G}_\alpha 1$, $\text{G}_\alpha 2$, $\text{G}_\alpha 3$, G_β , G_γ and G-protein β -subunits, although G proteins were detected in testes and sperm (Fig. 1*Bd*). The purification table (Fig. 1*c*) summarizes the steps involved in the chromatographic resolution of oscillogen activity and indicates that an overall $\sim 7,500$ -fold protein purification was achieved.

A mouse monoclonal antibody to the hamster 33K sperm protein was prepared to test further its specific correlation with oscillogen activity. Immunoblot analysis of the hydroxyapatite column fractions indicated that the monoclonal antibody J4 recognized the 33K protein present specifically in samples C and D which contain oscillogen activity (Fig. 2*a*, bottom), whereas

the monoclonal antibody C3 recognized the 29K protein that was enriched in samples A and B which lack oscillogen activity (Fig. 2*a*, top). Comparison with equivalent amounts of soluble extracts from other tissues indicated that the 33K protein is expressed in hamster sperm, but was not detectable in brain or liver (Fig. 2*b*), in agreement with previous microinjection studies indicating absence of oscillogen activity in brain and liver extracts¹². Isoelectric focusing in a pH 3–10 gradient indicated migration of the 33K protein with an isoelectric point (pI) of ~ 6 following immunoblot analysis with J4, which correlated exactly with fractions that displayed oscillogen activity in microinjection assays (Fig. 2*c*). Gel filtration chromatography showed that the 33K protein eluted as an oligomer in the void volume of a Superdex-75 column, again migrating specifically with oscillogen activity (Fig. 2*d*). The data using J4 suggest that the 33K protein may be

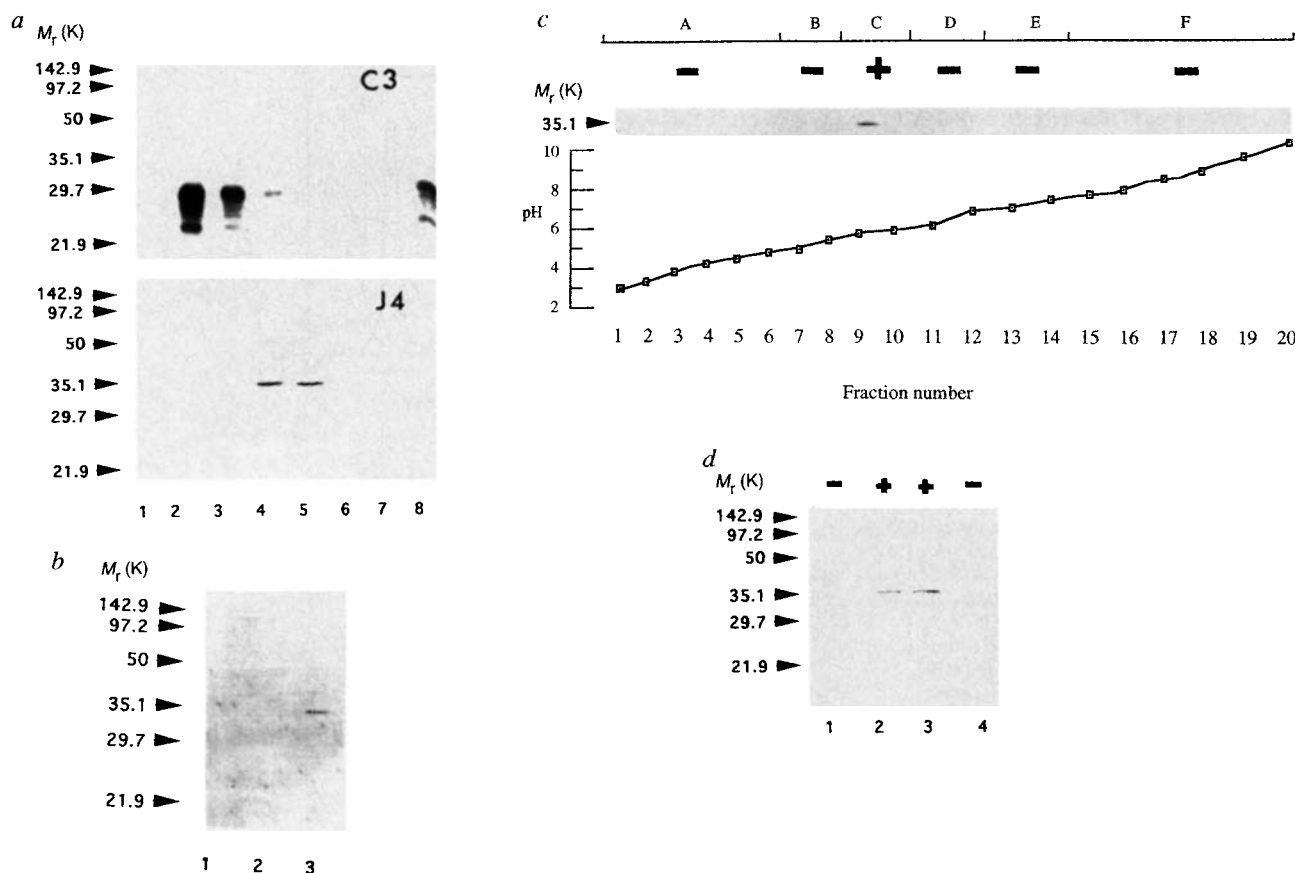


FIG. 2 Correlation of Ca^{2+} oscilligen activity with hamster-sperm 33K protein antibody, J4. *a*, immunoblot analysis of hydroxyapatite column samples with monoclonal antibody C3 to the 29K protein (top), and monoclonal antibody J4 to the 33K protein (bottom). Column flowthrough (lane 1), samples A-G (lanes 2-8). *b*, Immunoblot analysis of soluble extracts from hamster brain (lane 1), liver (lane 2) and sperm (lane 3) with J4 antibody. *c*, Isoelectric focusing profile of soluble sperm extracts on a pH 3-10 gradient and immunoblot analysis of fractions with J4 antibody. Fractions were pooled into samples A-F as indicated in inset. Oscilligen activity, observed only in sample C (denoted by plus symbol), was determined by microinjection into mouse eggs of samples A-F. *d*, size-exclusion chromatography of soluble sperm extracts and immunoblot analysis of fractions A-D (lanes 1-4) with J4 antibody. Oscilligen activity, observed in samples B and C (denoted by plus symbol), was determined by microinjection into mouse eggs of samples A-D.

METHODS. Monoclonal antibodies to the sperm 29K and 33K proteins were prepared from BALB/c mice immunized with samples C and D from the

hydroxyapatite column ($5 \times 50 \mu\text{g}$ at intervals of 3-4 weeks)²⁶. Monoclonal antibodies J4 (IgM subclass) and C3 (IgG subclass) were clones identified to be specific for the 33K and 29K sperm proteins, respectively. Hybridoma culture supernatants and ascites fluid were used for analysis of proteins electroblotted onto Immobilon (Millipore), and developed with horseradish peroxidase-conjugated anti-mouse immunoglobulins (Dako) and 3,3'-diaminobenzidine/ H_2O_2 (Sigma). Hamster brain and liver extracts were prepared as described previously¹². Isoelectric focusing of sperm extracts was performed for 5 h at 4°C with 2% ampholytes 3/10 (Biorad) using a Rotofor Preparative IEF Cell (Biorad). Fractions were pooled and concentrated (Centricon C-30) before assaying for Ca^{2+} oscilligen activity, as described in Fig. 1. Size-exclusion chromatography of sperm extracts was performed using a Superdex-75 gel filtration fast protein liquid chromatography (FPLC) column (Pharmacia) previously calibrated with M_r markers (Sigma). Samples A and D are the pooled fractions collected before and after the void elution volume of the column. Samples were concentrated and assayed as above.

a sperm-enriched protein, and further demonstrate that it consistently comigrates with the activity of a Ca^{2+} oscilligen.

Amino-terminal amino acid sequence analysis of the 33K hamster sperm protein allowed us to determine the primary structure by cDNA cloning and sequencing. A clone of 1.2 kb with a 3' poly(A) tail, isolated from a hamster testis cDNA library, contained an open reading frame that encoded a 289 amino acid polypeptide with a calculated M_r of 32,610, and predicted pI of 6.4 (Fig. 3a), consistent with our observations of the sperm-derived protein (Figs 1 and 2). Sequence identity of 53% was observed with a glucosamine-6-phosphate isomerase isolated from *Escherichia coli* (Fig. 3b). A role during fertilization for a mammalian sperm protein involved in hexose phosphate binding has not previously been shown, although an important hexose phosphate, inositol 1,4,5-trisphosphate (InsP_3), is the well-characterized second messenger that mobilizes intracellular calcium¹⁹.

The 33K protein recognized by monoclonal antibody J4 in different mammalian sperm was localized using indirect immuno-

fluorescence. In fixed and permeabilized hamster, boar and human sperm, J4 staining was observed to the equatorial segment region, with very weak staining in the sperm neck region (Fig. 4a, Ag, Ai). Labelling was to an intracellular site because non-permeabilized sperm did not show any significant staining (Fig. 4a, c). Specific staining also required sperm fixation, indicating the soluble nature of the 33K protein (data not shown). In contrast to J4, monoclonal antibody C3 to the 29K protein (Fig. 2a) stained a cap-like structure associated with the acrosome of hamster sperm, suggesting that it is not a component of the sperm oscilligen (Fig. 4a, e). C3 did not react with any structures in human or boar sperm (data not shown). This immunocytochemical data demonstrates that the 33K protein is localized at an intracellular site in the equatorial segment region of mammalian sperm.

The membrane proteins that bind sperm to eggs have been identified and sequenced in sea urchins and mammals^{7,10,11}. They appear to be involved in gamete fusion and not in triggering egg

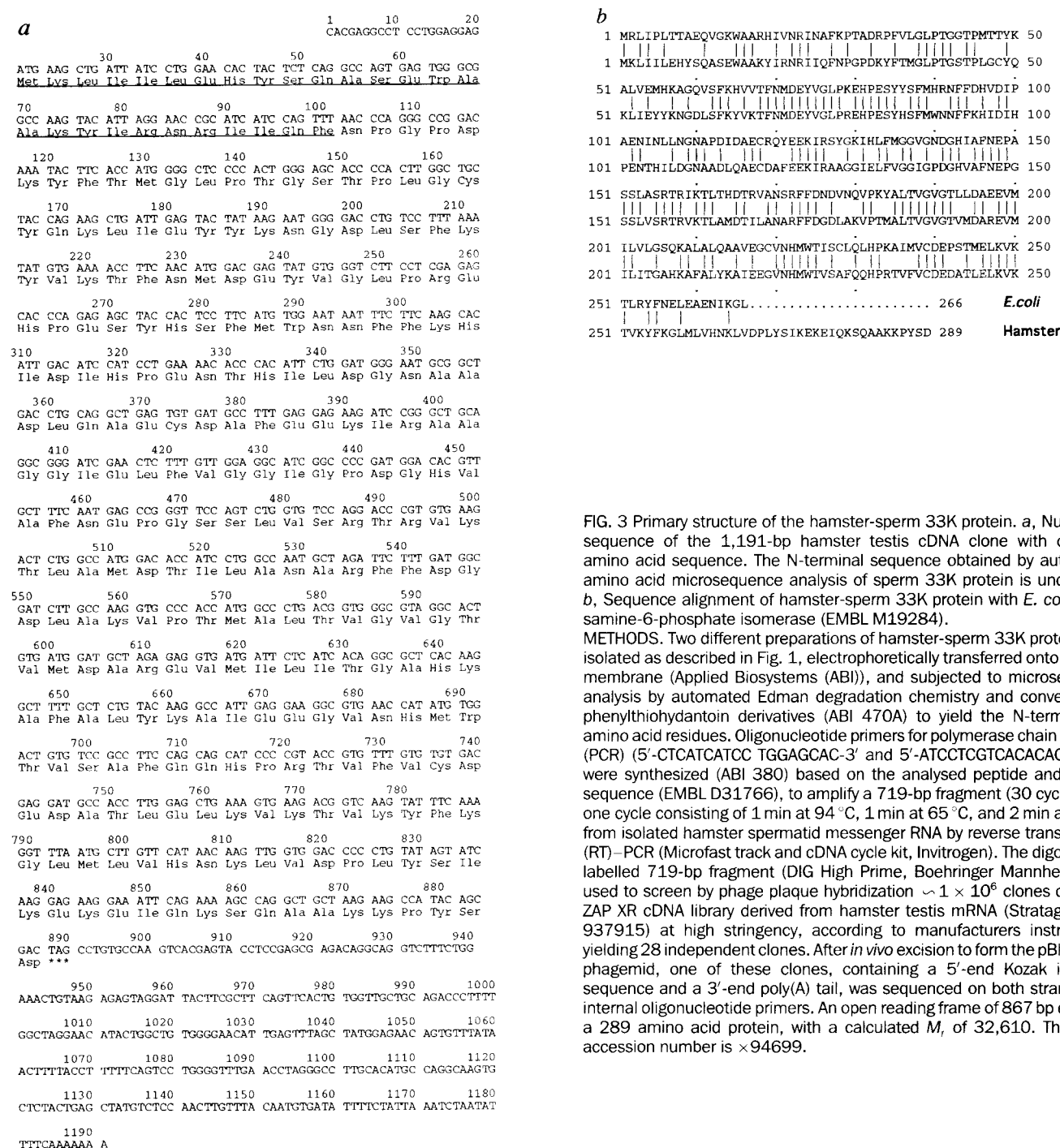


FIG. 3 Primary structure of the hamster-sperm 33K protein. **a**, Nucleotide sequence of the 1,191-bp hamster testis cDNA clone with deduced amino acid sequence. The N-terminal sequence obtained by automated amino acid microsequence analysis of sperm 33K protein is underlined. **b**, Sequence alignment of hamster-sperm 33K protein with *E. coli* glucosamine-6-phosphate isomerase (EMBL M19284).

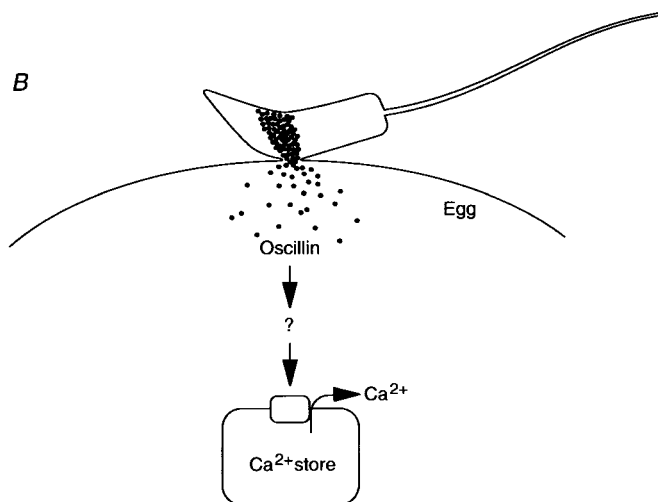
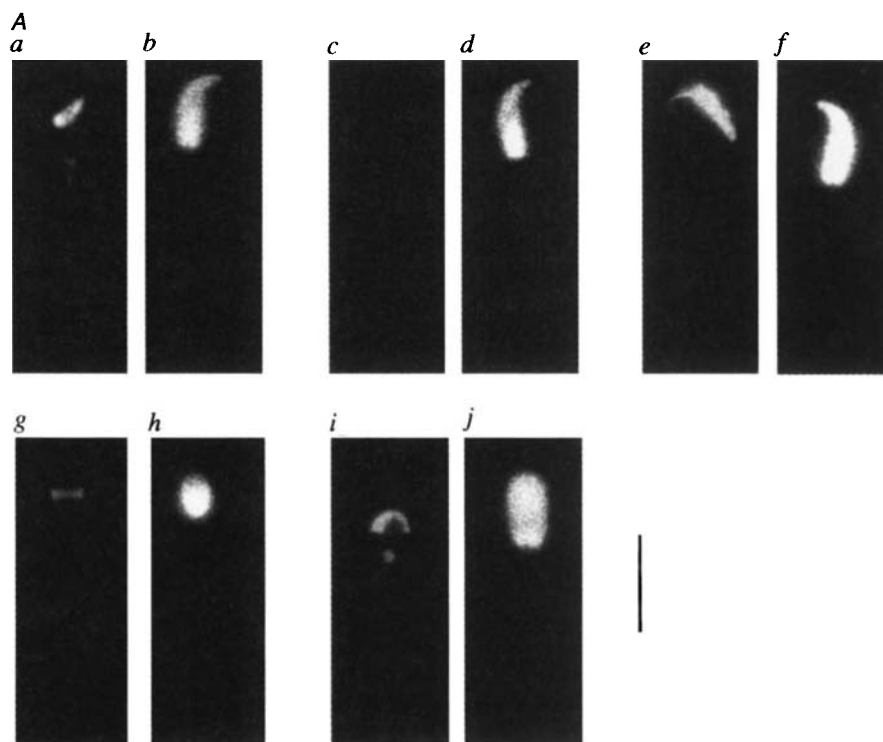
METHODS. Two different preparations of hamster-sperm 33K protein were isolated as described in Fig. 1, electrophoretically transferred onto Problott membrane (Applied Biosystems (ABI)), and subjected to microsequence analysis by automated Edman degradation chemistry and conversion to phenylthiohydantoin derivatives (ABI 470A) to yield the N-terminal 27 amino acid residues. Oligonucleotide primers for polymerase chain reaction (PCR) (5'-CTCATCATCC TGAGACAC-3' and 5'-ATCTCTGTACACACAAA-3') were synthesized (ABI 380) based on the analysed peptide and related sequence (EMBL D31766), to amplify a 719-bp fragment (30 cycles, with one cycle consisting of 1 min at 94 °C, 1 min at 65 °C, and 2 min at 72 °C) from isolated hamster spermatid messenger RNA by reverse transcriptase (RT)-PCR (Microfast track and cDNA cycle kit, Invitrogen). The digoxigenin-labelled 719-bp fragment (DIG High Prime, Boehringer Mannheim) was used to screen by phage plaque hybridization $\sim 1 \times 10^6$ clones of a Uni-ZAP XR cDNA library derived from hamster testis mRNA (Stratagene no. 937915) at high stringency, according to manufacturers instructions, yielding 28 independent clones. After *in vivo* excision to form the pBluescript phagemid, one of these clones, containing a 5'-end Kozak initiation sequence and a 3'-end poly(A) tail, was sequenced on both strands with internal oligonucleotide primers. An open reading frame of 867 bp encodes a 289 amino acid protein, with a calculated M_r of 32,610. The EMBL accession number is X94699.

activation^{6,7,10,11}. Previous reports of sperm-associated factors that trigger Ca^{2+} release or activate deuterostome eggs have shown them to be in the soluble fraction, but the active component(s) responsible for oscilligen activity have not been identified¹²⁻¹⁷. Our studies indicate that a new 33K sperm cytosolic protein, which is related to a prokaryote hexose phosphate binding protein, correlates with the ability of extracts to cause Ca^{2+} oscillations in mouse eggs. We suggest that this protein be called oscillin. After fusion of gamete membranes, entry of sperm oscillin into the egg to induce oscillations in intracellular Ca^{2+} may therefore constitute the primary intracellular signalling event that culminates in egg activation. As a consequence, we would expect sperm oscillin to be localized near the site where the sperm first fuses with the

egg, as the first Ca^{2+} transient in hamster fertilization occurs within seconds of membrane fusion^{3,6,8,20}. Our immunocytochemical data demonstrate that oscillin is concentrated specifically at an intracellular location in the equatorial segment of mammalian sperm, precisely the region where the sperm has been found to fuse with the egg in all mammals²⁰. As there is sufficient oscilligen activity in a single sperm equivalent^{12,18}, these data strongly support our hypothesis that oscillin, a soluble 33K sperm protein, is responsible for causing the Ca^{2+} oscillations that trigger egg activation at fertilization in mammals (Fig. 4B). In accordance with this hypothesis, the diffusion of oscillin out of sperm after intracytoplasmic sperm injection (ICSI) may help explain why this technique is successful in triggering the development of human

FIG. 4 Localization of J4 and C3 antigens in mammalian sperm stained with DNA-specific Hoechst dye (A), and proposed mechanism for triggering of calcium oscillations at mammalian fertilization (B). A, A single permeabilized hamster sperm labelled with J4 (a) and Hoechst dye (b). A non-permeabilized hamster sperm labelled with J4 (c) and Hoechst dye (d). A permeabilized hamster sperm labelled with mAb C3 (e) and Hoechst dye (f). A single permeabilized human sperm labelled with J4 (g) and Hoechst dye (h). A single permeabilized boar sperm labelled with J4 (i) and Hoechst dye (j). Scale bar, 10 μ m. B, Schematic representation of the localization of oscillin to the equatorial segment of the hamster sperm head, and its immediate diffusion into the egg cytoplasm on sperm-egg fusion to produce calcium oscillations by release from intracellular stores.

METHODS. Epididymal hamster sperm, ejaculated boar sperm (JSR HealthBred, Wiltshire, UK) and ejaculated human sperm were washed in phosphate buffered saline (PBS)^{12,14}. Sperm were fixed in 3% paraformaldehyde in PBS for 1 h at room temperature, washed again in PBS and smeared onto polylysine coated coverslips. Sperm were permeabilized with 0.5% Triton X100 in PBS for 10 min and sequentially treated with: mouse monoclonal antibodies J4 or C3 (0.2% ascites fluid in PBS); and then Texas red (1:20 dilution; Calbiochem) or FITC labelled (1:50 dilution; Sigma) anti-mouse immunoglobulin antibody with 1% BSA, 5% milk protein in PBS for 1 h each. Finally, sperm were stained with Hoechst 33342 at 10 μ g ml⁻¹ in PBS for 10 min. Slides were mounted in 50% glycerol and examined under epifluorescence.



eggs^{14,17,21}. Moreover, the reduced equatorial segment staining with J4 observed with some infertile males suggests that oscillin deficiency may be involved in some cases of male factor infertility (unpublished observations). Oscillin may mediate its effect by generating a Ca^{2+} -mobilizing second messenger, such as InsP_3 , within the egg^{8,22}, as InsP_3 is involved in triggering Ca^{2+} oscillations in somatic cells¹⁹. Alternatively, oscillin may operate by directly sensitizing Ca^{2+} release mechanisms within the egg^{13,18}. Because microinjection of sperm extracts also causes Ca^{2+} oscillations in neurons²³ and hepatocytes²⁴, it is possible that oscillin, or related proteins, may be involved in Ca^{2+} release in somatic cells. □

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Crystal structure of a G_A protein $\beta\gamma$ dimer at 2.1 Å resolution

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MANY signalling cascades use seven-helical transmembrane receptors coupled to heterotrimeric G proteins ($G_{\alpha\beta\gamma}$) to convert extracellular signals into intracellular responses¹. Upon nucleotide exchange catalysed by activated receptors, heterotrimers dissociate into GTP-bound G_α subunits and $G_{\beta\gamma}$ dimers, either of which can modulate many downstream effectors^{2,3}. Here we use multiwavelength anomalous diffraction data to solve the crystal structure of the $\beta\gamma$ dimer of the G protein transducin. The β -subunit is primarily a seven-bladed β -propeller that is partially encircled by an extended γ -subunit. The β -propeller, which contains seven structurally similar WD repeats, defines the stereochemistry of the WD repeat and the probable architecture of all WD-repeat-containing domains. The structure details interactions between G protein β - and γ -subunits and highlights regions implicated in effector modulation for the conserved family of G protein $\beta\gamma$ dimers.

Rod transducin, $G_{\alpha\beta\gamma}$, is one of the best studied G proteins, coupling photoactivated rhodopsin to the visual signalling cascade. Here we report the crystal structure of the transducin $\beta\gamma$ dimer, $G_{\beta\gamma}$, solved by multiwavelength anomalous diffraction (MAD) and refined at 2.1 Å resolution (Table 1 and Fig. 1a). $G_{\beta\gamma}$ belongs to the β -propeller family of proteins⁴ whose members contain 4–8 antiparallel β -sheets resembling the blades of a propeller (Fig. 1b). The β -propeller of $G_{\beta\gamma}$ consists of seven β -sheets, each containing 4 antiparallel strands radiating outwards from a central core with approximate sevenfold symmetry. The inner strands of each sheet run roughly parallel to the central axis and form the walls of a water-filled tunnel, whereas the outer strands tilt as they radiate away from the centre.

The approximate sevenfold symmetry of the β -propeller is reflected in the amino-acid sequence, which contains seven WD repeats (Fig. 1b–e). The WD-repeat motif is approximately 40 amino acids long and contains a number of conserved amino acids, including a Trp-Asp dipeptide—the ‘WD’ that frequently terminates the repeat. Initially identified⁵ by sequence homology within transducin’s G_β subunit and CDC4, sets of 4–8 WD repeats have since been found in the sequences of about 40 other eukaryotic proteins^{6–8}.

A single WD repeat does not correspond to a stably folded domain, or even a single β -sheet; instead, the WD repeat begins at the turn between the third and fourth (outermost) strands of a β -sheet and ends at the equivalent position in the following β -sheet (Fig. 1b–e). The outer strand of the seventh β -sheet is provided by the N-terminal strand of the first WD repeat, thus linking the beginning and end of the β -propeller. Although some of the loops and turns between strands differ in length by as many as five amino acids, these deviations are exceptional, and occur only three times among the 28 connecting segments in the β -propeller of $G_{\beta\gamma}$. However, large insertions between or within WD repeats do occur in other sequences; for example, the phosphorylation of a 41-amino-acid insertion within the sixth repeat of the yeast G_β subunit (Ste4p) is necessary for adaption of the mating

response^{9,10}. As the number of WD repeats in proteins and the number of blades in β -propellers span the same range (4 to 8), it is likely that other proteins containing WD repeats will also form β -propellers.

Within each WD repeat in $G_{\beta\gamma}$, conserved residues preserve a common set of interactions which span the breadth of the repeat and position adjacent blades of the β -propeller (Fig. 1b–e). The interactions are characterized by a large hydrophobic residue, typically tryptophan, buried in a non-polar environment between sheets and interacting, within the same repeat, with a Asp-His-Ser/Thr structural triad of residues reminiscent of the catalytic triads in serine proteases. The only invariant residue among the seven repeats in $G_{\beta\gamma}$ is the aspartate of the structural triad which is located in a tight turn between the middle two strands of each sheet (position 35 in Fig. 1d). The carboxylate oxygens of this aspartate hydrogen-bond to main-chain amides both in the tight turn and directly following the conserved histidine in the loop connecting the first and second strands of the same repeat (Fig. 1c). This hydrogen-bonding arrangement stabilizes and effectively couples the tight turn of one β -sheet to the outer strand of the previous β -sheet. As a consequence, the conserved histidine (position 13) is optimally oriented and polarized by the aspartate. The polarized histidine thus readily accepts a hydrogen bond from the side chain hydroxyl of a serine or threonine residue in the second strand of the β -sheet (position 31), which in turn accepts a hydrogen bond (~ 2.8 Å) from the side chain of the buried tryptophan in the third strand of the β -sheet (position 41). This spatial arrangement of conserved residues preserves a network of interactions identical in four WD repeats of $G_{\beta\gamma}$. However, the conserved residues are not invariant, leading to modest structural differences in repeats 2, 5 and 6.

Neither the Trp nor the Asp found at the end of the third strand of a sheet, from which the WD repeat is named, are as well conserved among known repeats⁶ as the Asp and His of the structural triad. Within a repeat of $G_{\beta\gamma}$, the trademark Trp (or another aromatic residue) is buried by contact with up to 5 other residues. The residue following this Trp, normally Asp or Asn, serves to end the last strand of the repeat, stabilizing the turn between the third and fourth strands of a sheet through hydrogen-bonding and electrostatic interactions that vary in different repeats.

Proteins with β -propellers exist in both prokaryotes and eukaryotes, and while their structures can be strikingly similar, their functions are widely disparate. For instance, galactose oxidase is a fungal, seven-bladed propeller (Fig. 1f) that uses free radicals to oxidize primary alcohols¹¹. Although most known β -propellers contain weak repetitive sequence motifs, no connection with WD repeats has been noticed. But on closer inspection, the most conserved portions of the repeats in galactose oxidase have a degree of sequence similarity with equivalent regions in the WD repeats of $G_{\beta\gamma}$ (data not shown), meriting further study into a possible evolutionary relatedness between WD-repeat proteins and other β -propellers.

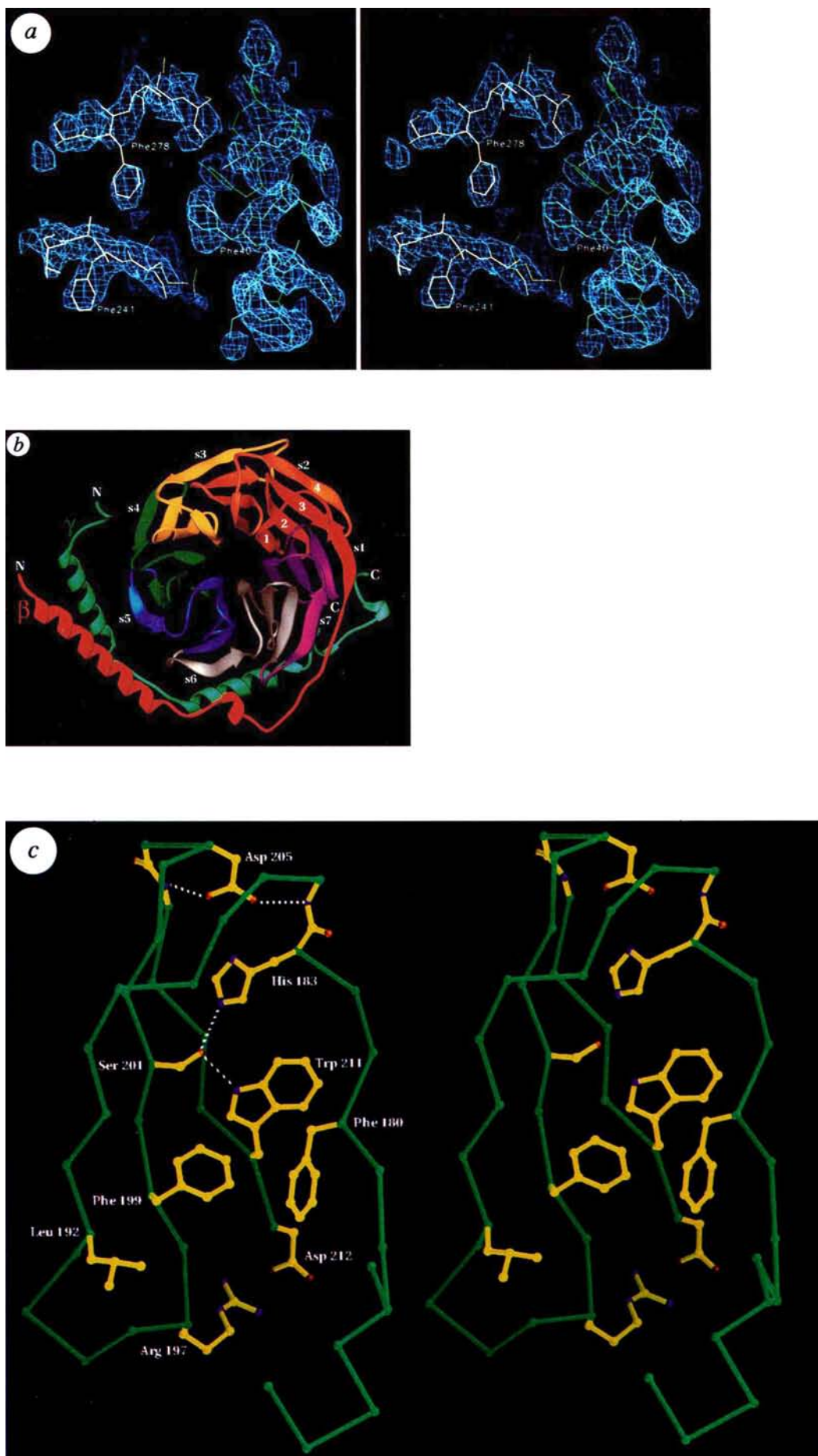
Normally, G_β and G_γ are tightly associated, and expressing them separately leads to unstable G_β subunits and unfolded G_γ subunits¹². The structure of $G_{\beta\gamma}$ explains this interdependence (Fig. 1b). G_γ forms extensive, mainly hydrophobic interactions with G_β and binds in an extended conformation completely lacking intrachain tertiary interactions (Fig. 2b). As predicted^{13,14}, the N-terminal helix of G_γ forms a parallel coiled-coil with the N-terminal helix of G_β . The coiled-coil interface is mainly hydrophobic and is further stabilized by solvent-exposed ion pairs.

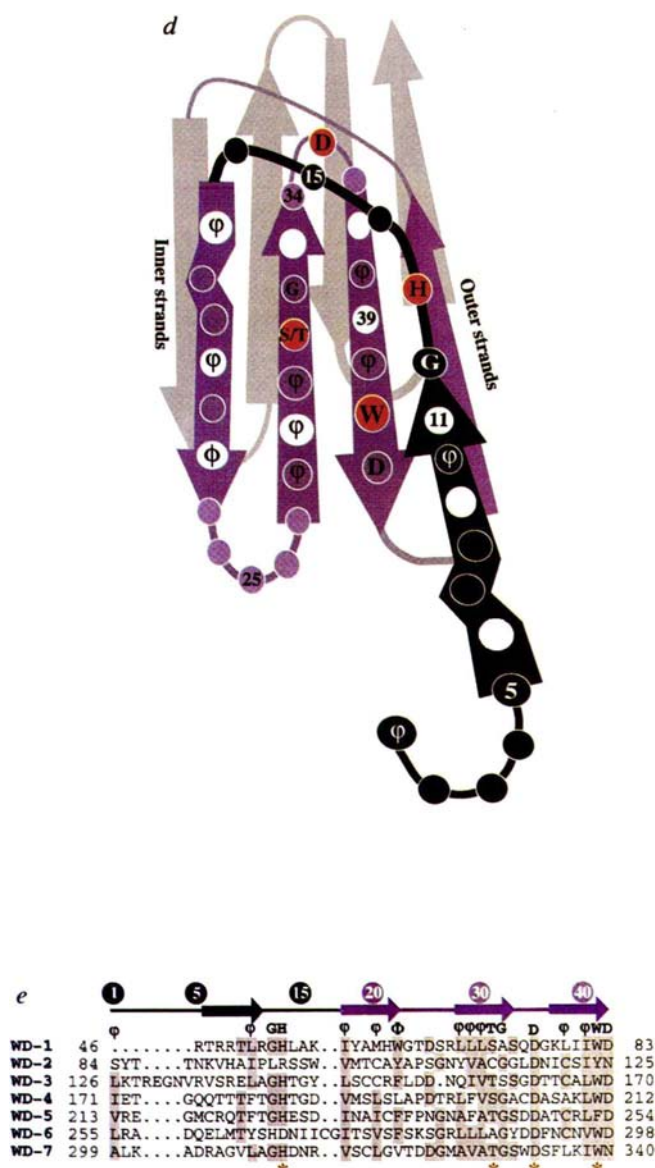
In spite of the small number of interactions between the coiled coil and the β -propeller, the position of the coiled coil is remarkably constant in all 4 protomers of the asymmetric unit. Many of the interactions that stabilize the coiled coil’s position relative to the β -propeller depend on Asp258 in the sixth sheet of G_β . Asp258 interacts with two residues in the region linking the coiled coil to the β -propeller—Arg22 in G_β and Arg30 in G_γ . The orientation of Asp258 is further buttressed by Gln220 in the

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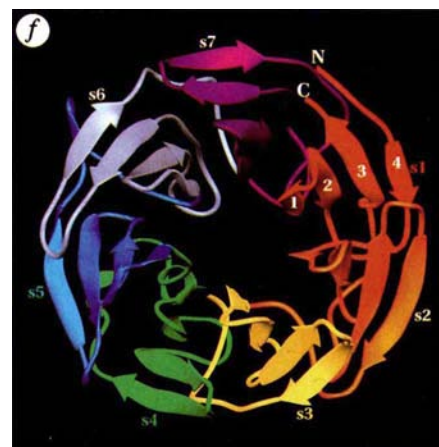
FIG. 1 Overall structure of heterodimeric G_{ly} . **a**, Stereo view of experimental, solvent-flattened electron-density map based exclusively on MAD phasing ($2.8\text{ \AA}$, 1.2σ) and centred on Phe 40 of G_{ly} . Residues from G_{ly} are yellow; G_{tr} residues are green. **b**, Ribbon²⁹ drawing viewed down the central tunnel and coloured to highlight the 7 WD-repeats of G_{ly} . Sheets and strands are labelled numerically; sheets contain the prefix 's'. **c**, Ball-and-stick model of the fourth WD repeat in G_{ly} . View is roughly perpendicular to the central tunnel and the plane of fourth β -sheet. C α main chain is green, structurally important main-chain and side-chain atoms are yellow except for nitrogen (blue) and oxygen (red). White dotted lines depict hydrogen bonds. Within the structural triad, hydrogen bonds between Asp and His side chains are unlikely as donor/acceptor atoms vary in distance ($\sim 3.0\text{--}3.6\text{ \AA}$) and geometry. Superposition of C α atoms in the 4 strands of the first WD repeat of G_{ly} with equivalent atoms in repeats 2–7 yields r.m.s. deviations of: 1.3, 0.6, 0.6, 0.6, 1.7 and $1.0\text{ \AA}$, respectively. **d**, In roughly the same orientation as that shown in **c**, schematic of the consensus WD repeat of G_{ly} labelled with conserved residues and showing the relationship between consecutive WD repeats. Red highlights the structural triad and the trademark Trp. Strands from three consecutive β -sheets are differentiated by black, blue and grey. A single WD repeat is delimited by labelled residues. White and red circles represent residues pointing towards the viewer. **e**, Alignment of the seven WD repeats in G_{ly} . Four or more conserved residues at equivalent positions are highlighted grey. Sites with 5 or more similar residues form the consensus sequence shown above the alignment in bold; ϕ designates any hydrophobic residue and ψ designates aromatic residues; T designates Thr or Ser. Circled numbers and coloured arrows correspond to positions and secondary structure, respectively, in the consensus WD repeat in **d**. The structural triad and the trademark Trp are indicated by red stars. **f**, 'Ribbons' depiction of the β -propeller domain of galactose oxidase¹¹ showing the structural similarity to the β -propeller of G_{ly} . Coordinates of galactose oxidase obtained from the Protein Data Bank, id. code 1gog.





fifth sheet of G_{β} . Each arginine also forms a hydrogen bond with a main-chain carbonyl, further helping to position the coiled coil. As the residues involved in these interactions are conserved in G_{β} and G_{γ} subunits (Fig. 2), the position of the coiled coil with respect to the β -propeller is likely to be relatively constant throughout the heterotrimeric G-protein family.

Phe 40 and Phe 64 in G_{γ} (Fig. 2b) form extensive hydrophobic interactions with G_{β} . Phe 40 inserts into a hydrophobic cleft between sheets 6 and 7 and is flanked by further hydrophobic interactions between the second helix of G_{γ} and the β -propeller. Phe 64 is a well defined landmark along the C-terminal portion of G_{γ} , interacting with many residues in sheets 1 and 7. Poor electron density for residues 66–68 and the proteolytic susceptibility of Lys 68 suggest that the final six residues and the farnesyl moiety,



linked through a sulphur–ether bond to C-terminal methylated Cys 71, do not form a stable conformation in the absence of membranes and/or rhodopsin.

Although not all $G_{\beta\gamma}$ dimers are possible, there are a large number of stable combinations between the five mammalian G_{β} subunits and the eight mammalian G_{γ} subunits. Combinatorial dimerization raises the possibility of differential effector regulation by unique $G_{\beta\gamma}$ dimers, and studies addressing this issue^{15–19} have localized regions on both subunits which are important for defining dimerization specificity. For example, chimaeras of $G_{\gamma 1}/G_{\gamma 2}$ identified Phe 40 of G_{γ} as especially important for discriminating between dimer combinations²⁰. Whereas other G_{γ} subunits with smaller side chains at this position will bind G_{β} , G_{γ} will not bind other G_{β} subunits. One possibility is that Phe 40 cannot insert into the presumably similar but smaller and conserved cavities of other G_{β} subunits. Of the residues that create this cavity, only position 300 is variable, and substitution at this site could reduce the cavity enough to prevent insertion of Phe 40. G_{β} chimaeras having variable coiled coils failed to impart specificity to G_{β}/G_{γ} dimerization²¹. This is consistent with the interface of the coiled coil of $G_{\beta\gamma}$ containing mainly hydrophobic residues which are conserved in all G_{β} and G_{γ} subunits. Sheets 5 and 6 of $G_{\beta\gamma}$ participate in a large number of interactions at the β/γ interface, and the structure supports studies^{16,21} of chimaeric G_{β} subunits which localized important dimerization determinants to this region (i.e., residues 214–303 of G_{β}).

$G_{\beta\gamma}$ dimers were originally shown to activate cardiac K^+ channels²² and have since been observed modulating a diverse array of downstream effectors³. Studies using extensive saturation mutagenesis^{23,24} identified two clusters of dominant-negative, single-site substitutions in yeast $G_{\beta\gamma}$, strongly implicating these regions in effector modulation (Fig. 3). One of these regions (sites 135 and 138 in G_{β} at the outer strand of the second sheet) is adjacent to, but does not overlap, the G_{α}/G_{β} switch interface (see accompanying paper²⁵). The remaining six sites are on the opposite side of G_{β} . Three of these residues are from G_{β} and form a contiguous, exposed surface near the segment that links the coiled coil to the β -propeller. The other three residues implicated in effector modulation are from G_{γ} and are buried on the side of the coiled coil facing the β -propeller. Whereas the region linking the coiled coil to the β -propeller has a similar structure in all four dimers in the asymmetric unit, this region exhibits the largest structural differences when compared to the $\beta\gamma$ dimer in the heterotrimeric $G_{\alpha\beta\gamma}$ complex. Considered with the consistently higher thermal parameters of the coiled coil, these structural differences suggest that this region is uniquely flexible and raises the possibility that the coiled coil may change position upon binding effectors. Of the 13 least-conserved sites among the five eukaryotic G_{β} subunits, 11 lie within the coiled coil or flank the G_{γ} subunit.

TABLE 1 Summary of crystallographic analysis

Data collection statistics									
	$\Delta f' / \Delta f''$	NSLS beamline*		Reflections (observed/unique)	Resolution limit (Å)	$R_{\text{sym}}^{\dagger}$ (%)	$R_{\text{anom}}^{\ddagger}$ (%)	Data coverage (%)	
Native		X25C		258,531/88,015	2.1	8.8		96.0	
Gd ³⁺		X4A							
$\lambda_1 = 1.7117 \text{ Å}$	−28.02/3.63			185,353/38,045	2.8	11.4		90.6	
$\lambda_2 = 1.7110 \text{ Å}$	−19.95/14.7**			185,931/68,650§	2.8	10.1	9.9	90.7	
$\lambda_3 = 1.7104 \text{ Å}$	−18.60/10.59			184,501/68,471§	2.8	9.9	7.1	90.4	
Phasing statistics									
Resolution limit (Å)	16.09	9.59	6.83	5.30	4.33	3.66	3.17	2.80	overall
Phasing power ¶									
Gd ³⁺ (λ_2)	0.60	1.10	1.40	1.36	0.86	0.60	0.56	0.45	0.64
Anomalous	0.54	0.71	0.89	1.02	1.01	0.86	0.73	0.59	0.77
Gd ³⁺ (λ_3)	0.70	1.01	1.29	1.42	0.93	0.70	0.71	0.61	0.78
Anomalous	0.59	0.81	0.89	0.96	0.82	0.69	0.58	0.47	0.63
Mean figure of merit	0.51	0.68	0.69	0.64	0.47	0.39	0.34	0.24	0.25
Refinement statistics									
	Resolution (Å)		<i>R</i> factor		Free <i>R</i> factor#		<i>N</i> reflections		
Data with <i>F</i> > 2σ	6.0 – 2.1		0.191		0.300		71,169		
All data	6.0 – 2.1		0.211		0.315		83,981		
R.m.s. deviations	bond lengths: 0.011		Bond angles: 1.897		improper dihedrals: 1.414				

$G_{\beta\gamma}$ was purified from bovine rod outer segments using existing protocols²⁶. Disc membranes were washed, and the heterotrimeric G protein was released from the membranes with GTP γ S. $G_{\beta\gamma}$ was digested with endoproteinase Lys-C (endo-LysC) together with $G_{\alpha\gamma}$ -GTP γ S (for 40 mg $G_{\alpha\beta\gamma}$: 20 units of endo-LysC for 36 h at 4 °C) and then separated from $G_{\alpha\gamma}$ using a Hi-trap Blue column (Pharmacia). Endo-LysC cleaves C-terminal of Lys 68 in $G_{\alpha\gamma}$, removing 3 amino acids and the farnesyl moiety. $G_{\alpha\gamma}$ is unaffected by endo-LysC. $G_{\beta\gamma}$ was purified on a Mono-Q column (Pharmacia), concentrated using Centrprep-10s (Amicon), and stored at $> 90 \text{ mg ml}^{-1}$ at -80 °C in 5 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 15 mM NaCl and 40% (v/v) glycerol. A typical preparation, starting with 1,600 retinæ, yielded ~20 mg of $G_{\beta\gamma}$. To crystallize $G_{\beta\gamma}$, 2 μ l of 15–20 mg ml⁻¹ $G_{\beta\gamma}$ in 1 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 5 mM NaCl, 15 mM β -mercaptoethanol and 5–7.5% (v/v) glycerol was mixed 1:1 with well solution (10 mM fumarate, pH 4.2, 10 mM MgSO₄, 2 mM GdCl₃, 5% (v/v) glycerol, 5% (w/v) polyethylene glycol 4000, 15 mM β -mercaptoethanol) and equilibrated in a hanging drop at 4 °C. Crystals were harvested at 3–4 weeks, having reached typical dimensions of 50 $\times$ 50 $\times$ 200 μ m. Crystals were transferred to (10 mM fumarate, pH 4.2, 10 mM MgSO₄, 10% (v/v) glycerol, 5% (w/v) polyethylene glycol 8000, 15 mM β -mercaptoethanol), and the glycerol concentration was incrementally increased to 40% (v/v). Gd³⁺-derivatized crystals were prepared by transfer into a solution with 25 mM GdCl₃, and equilibrated for 12 h. All crystals were flash-frozen and stored in solid propane. Crystals are of the orthorhombic space group $P2_12_12_1$ ($a = 85.1 \text{ Å}$, $b = 94.0 \text{ Å}$, $c = 194.7 \text{ Å}$). MAD data, near the Gd edge, were collected at NSLS beamline X4A. Owing to time constraints, only three wavelengths were collected: λ_1 at the $\Delta f'$ minimum, λ_2 at the $\Delta f''$ maximum, and λ_3 at a point just beyond the $\Delta f''$ peak. No attempt was made to collect 'reverse beam' data. Six Gd³⁺ sites were identified in an anomalous difference Patterson of the λ_2 data set and 3 additional sites were located in anomalous difference Fourier maps using the preliminary MAD phases. The data from λ_2 and λ_3 were treated as independent derivatives, and for each of the nine Gd³⁺ sites, both the isomorphous and anomalous occupancies were refined against the λ_1 data set with Friedel pairs merged. The resolution of the solvent-flattened, experimental map, was limited by the long wavelength at the Gd edge (~1.70 Å). However, this map was of sufficient quality to allow much of the main chain to be placed into density. There are four pseudo-222 related heterodimers per asymmetric unit. The initial model was used to define the non-crystallographic symmetry (NCS) operations necessary for NCS averaging and phase improvement. The model was completed using symmetry-averaged and partial model combined maps. Resolution was extended to 2.1 Å using data from a non-derivatized crystal collected at NSLS-X25 beamline and the model was refined without NCS restraints using X-PLOR²⁷ version 3.1. In the asymmetric unit, the model contains: the complete amino-acid chain for all 4 $G_{\beta\gamma}$ subunits, residues 1–65 for two $G_{\alpha\gamma}$ subunits, residues 11–65 for the other two $G_{\alpha\gamma}$ subunits, (68 amino acids for proteolysed $G_{\alpha\gamma}$ and 71 amino acids for full-length) and 732 water molecules. Pairwise differences between corresponding C α atoms in the four $G_{\beta\gamma}$ models range from 0.950–1.185 Å. Differences in corresponding C α atoms between the four $G_{\beta\gamma}$ model and the $G_{\beta\gamma}$ component in the $G_{\alpha\beta\gamma}$ complex range from 0.82–1.03 Å. All data were reduced and scaled using the programs DENZO and SCALEPACK (Z. Otwinowski); phases were refined with MLPHARE (Z. Otwinowski, modified by G. Van Duyne); and the program DPHASE (G. Van Duyne) was used for solvent flattening, partial model combination, and NCS phase improvement. The structure was traced using the programs FRODO and O (ref. 28).

* National Synchrotron Light Source beamlines.

$\dagger R_{\text{sym}} = \sum |I_h - \langle I_h \rangle| / \sum I_h$ where $\langle I_h \rangle$ is the average intensity over symmetry equivalents.

$\ddagger R_{\text{anom}} = \sum ||F_h^+| - |F_h^-|| / \sum (|F_h^+| + |F_h^-|) / 2$.

§ Friedel pairs separate.

|| Phasing power = $\sum |F_H| / \sum |F_{\text{PHobs}}| - |F_{\text{PHcalc}}|$.

¶ Anomalous phasing power = $\sum |F_H^+| / \sum |AD_{\text{obs}}| - |AD_{\text{calc}}|$.

10% of data.

** Estimated from refined anomalous occupancy of Gd³⁺ sites at this wavelength.

FIG. 2 Sequence and secondary structure features of G_{β} and G_{γ} subunits. a, Alignment based on the homology of 11 G_{β} subunits and grouped to emphasize the seven WD repeats defined by the structure of $G_{\beta\gamma}$. Residues that contact $G_{\alpha\gamma}$ are shown in grey; those that contact $G_{\alpha\gamma}$ are red (see accompanying paper²⁵). Residues important for effector function in yeast G_{β} localize in two areas and are starred blue²⁴. Substitutions in yeast G_{β} which disrupt G_{α} interaction are marked with red stars³⁰. SWISS-PROT accession numbers from top to bottom are: P04901, P11016, P16520, P29387, NA, P17343, P12322, P26308, P36408, P29829, P18851, where NA indicates not available and EMBL accession number L34290. b, Alignment based on the homology of 12 G_{γ} subunits, emphasizing contacts between G_{α} and G_{β} shown in grey. Blue asterisks indicate sites necessary for effector activity in yeast²³ and the farnesylated, methyl-esterified Cys is boxed.

SWISS-PROT release 31.0 accession numbers from top to bottom are: P02698, Q08447, NA, P30670, NA, P16874, NA, P29798, P30671, P38040, Q01821, P18852, where NA indicates none available and taken from ref. 15. Lower panel indicates specific hydrophobic interactions between G_{α} and G_{β} are shown in black, specific ionic interactions in red. Genetics Computer Group Wisconsin package v. 8 was used for initial alignments and then subjectively modified. Per cent solvent-accessibility histograms were based on 25% increments using ACCESS³¹ and secondary structure according to DSSP³². Specific interactions are less than 4 Å apart and visually inspected for reasonable stereochemistry. Residues forming intersubunit contacts either bury >20% of their solvent accessible surface area or make specific interactions.

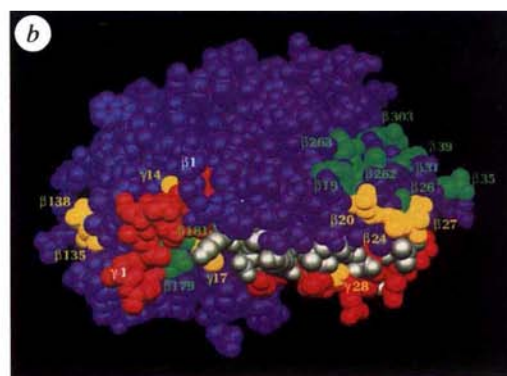
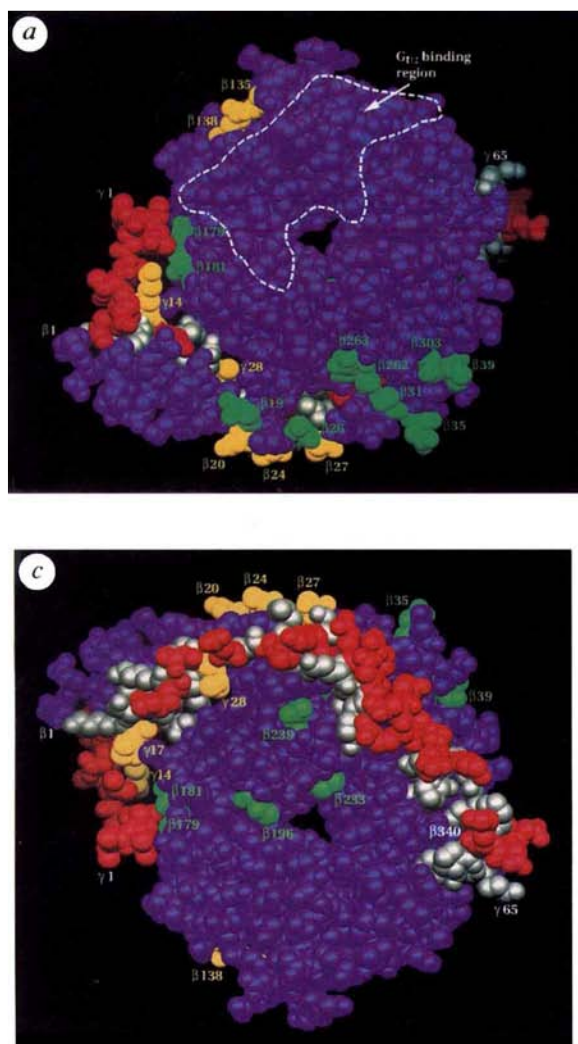


FIG. 3 Location of non-conserved residues and sites implicated in effector signalling in $G_{\beta\gamma}$. *a*, van der Waals space-filling model in the same orientation as in Fig. 1*b* and coloured to highlight functionally important and non-conserved residues that cluster along one side of the dimer and lie alongside $G_{\beta\gamma}$. $G_{\beta\gamma}$ is blue, except for the non-conserved residues which are green. For G_{γ_1} , conserved residues are grey and non-conserved residues red. Residues implicated in yeast effector signalling are yellow. Residues that change by > 20% in their solvent accessibility upon binding G_{α} are conserved and encompassed by a white, dotted line (accompanying paper). Non-conserved residues in G_{γ_2} are defined as tolerating 4 or more different residues at equivalent positions among the 5 eukaryotic G_{γ} subunits. Conserved positions in G_{γ} have at least six identical or seven homologous residues at equivalent sites among the mammalian G_{γ} subunits. *b*, View similar to that in *a*, rotated $\sim 90^\circ$ about an axis perpendicular to the central tunnel. *c*, View similar to that in *a*, rotated $\sim 180^\circ$ about an axis perpendicular to the central tunnel and highlighting the solvent-exposed and non-conserved residues of G_{α} .

The non-conserved residues in G β subunits could augment the high degree of variability found throughout the solvent-exposed surface of G α (Fig. 3c) in defining effector specificity. $\square$

Note added in proof: After this paper was submitted, Wall *et al.* (Cell 83, 1047–1058, 1995) published a partially refined structure of G_{1β1γ1γ2} showing a similar overall architecture for the G_B-dimer.

Received 1 December 1995; accepted 2 January 1996.

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- Received 1 December 1995; accepted 2 January 1996.
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ACKNOWLEDGEMENTS. We thank C. Ogata and W. Hendrickson at X-4A; R. Sweet and L. Berman at X-25 for access to and help with beamlines at the NSLS, Brookhaven National Labs; and members of P.B.S.'s laboratory for assistance during data collection and technical help. This work was supported by grants from the NIH to P.B.S. and H.E.H. H.E.H. was also supported by the American Heart Association. D.G.L. and J.S. are Damon Runyon-Walter Winchell postdoctoral fellows. Coordinates of $G_{\beta\gamma}$ will be submitted to the Protein Data Bank.

A guide to scientific products

Dipping the oars into some fresh waters, product review offers product information on astronomy and oceanography, as well as our standard complement, including electroporation cuvettes and a direct blotting electrophoresis system.

NETWORK Computing Devices offers X terminals for use by astronomers and scientists to calibrate and analyse astronomical data. The manufacturer states that these have been useful during the migration from VMS to UNIX, and that the terminals used with the older VAX/VMS machines can now be used with the new UNIX servers. Astronomers using the X terminals can access over eighty applications in the Starlink software collection, covering a wide range of astronomical data-reduction and analysis techniques. The system is designed to allow the sharing of resources, without the expense of providing a UNIX workstation at every desk.

Reader Service No. 100

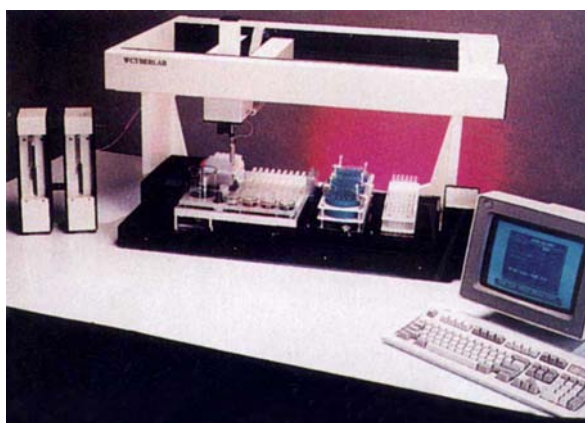
Chelsea Instruments has added a third towed vehicle, Nv-Shuttle, to its range of highly reliable, versatile oceanographic vehicles. The shuttle has been designed to meet the requirements of a low cost, large payload, towed undulating vehicle while retaining the hydrodynamic performance of its sister vehicle, Aquashuttle. The manufacturer states that the vehicle has been flown on extensive sea trials in the Atlantic, Mediterranean and Pacific over a three-year period and has been shown to be a stable and robust vehicle. Two versions are available with a choice of 90 or 120 litres of instrumentation space. Both vehicles are capable of carrying a suite of instrumentation for the measurement of parameters such as depth, temperature, salinity, chlorophyll, fluorescence, turbidity, transmittance, bioluminescence, nutrient, redox and dissolved oxygen. The body design is said to allow ease of access of total payload. The shuttle can be towed from research vessels and ships of opportunity at speeds of up to 26 knots, to operational depths of 150 m. It can be launched and recovered while the vessel is under way, by non-scientist crew members with minimal training. The Nv-Shuttle is self-contained, with an independent power source and an integral servo controlled system driving the elevator. The required undulation path is adjustable and the depth and pitch parameters are stored in solid-state, onboard memory. These parameters are set over

the RS422 communications interface and can be set either before deployment or during development in real time.

Reader Service No. 101

Laboratory essentials

Columbus Instruments' **modular, miniature exerciser for mice** can be expanded from single to multiple lanes to exercise up to eight mice. The device has been designed to provide a sufficient runway length and at the same time minimize the air volume inside the exercise chamber for fast response of VO_2/VCO_2 metabolic measurements. The first master unit contains a single lane with an electrical motor and motor controller. Additional, less expensive lanes can be coupled mechanically to the master unit.



The Cyberlab C-200 sample preparation workstation.

Up to eight lanes can be operated by a single motor and controller. The modular exerciser can be equipped with electric stimulus or air puff stimulus for each exercise lane. Each lane can be made air-tight and used in conjunction with the company's Oxymax metabolic computer for studying animal oxygen consumption during exercise. A miniature electric fan is located inside the animal compartment to ensure good air circulation. The belt speed is precisely controlled in the range 0 to 2 m s⁻¹ and is displayed on a digital meter.

Reader Service No. 103

Cyberlab has announced the design of its new C-200 sample preparation workstation for use in general and research laboratories. These workstations can be

used for filling, pipetting, sampling and manipulating a variety of substances into a variety of containers. Various gripping tools transport samples for dispensing, weighing, bar-code reading, vortexing, heating, cooling, as well as other functions. It offers up to eight tools and interchangeable heads — pipetting heads can be equipped with either fixed or standard disposable tips for zero carry-over. The open design of the workstation is said to allow easy integration into large robotic systems.

Reader Service No. 102

The Leica DM IRB/E **inverted research microscope**, now has the optional feature of 100 per cent light to the side-access photo port. New stand configurations are available with a sideport beamsplitter of 100 per cent photo/zero per cent visual or zero per cent photo/100 per cent visual. This newest member of the DM R family of modular research microscopes is designed for routine investigations and research in biology and medicine, particularly cellular and genetic research. The manufacturer states that it is suited for micromanipulation applications, such as production of single cultures, isolation of bacteria and *in vitro* fertilization.

Reader Service No. 104

Hewlett-Packard has announced that its HP 3D capillary electrophoresis (CE) system now can perform capillary electrochromatography (CEC), a new technique that offers separation efficiencies stated to be 2–3 times better than conventional HPLC. It is also said to allow the separation of compounds with very closely related structures. Typical applications of CEC are the separation of neutral compounds with low molecular weight, for example drug candidates or drug precursors. The isolated fraction can be used for further off-line analysis. A capillary electrophoresis–mass spectrometry adapter kit enables the system to be interfaced to an HP 8959 ESI-MS or to electrospray ionization mass spectrometers from other manufacturers. The kit includes an MS interface cassette, a nonmetal alignment interface for 360- μm absorbance capillaries and a CE-MS capillary. Parts required to adapt

NEW ON THE MARKET

a mass spectrometer, such as make-up flow pump, CE-MS probe or other items, must be provided by the user.

Reader Service No. 105

Hellma now offers a complete line of fluorescence cells for the characterization of the fluorescent properties of fluids. The manufacturer states that these rectangular, high-quality, nonfluorescing glass and quartz cells are particularly suited for fluorescence experiments. Cells with metal coated windows are available, and are designed to enhance



Hellma's line of fluorescence cells.

the yield of fluorescent light. The window opposite the one which transmits the incoming radiation, as well as one window perpendicular to the transmitting window are metal coated on the exterior and then covered with a layer of protective black enamel to guard against scratches. A comprehensive catalogue of fluorescent cell products is also offered.

Reader Service No. 106

Medical Systems has introduced its new Micro-Incubator used to **maintain cells or tissue on the stage of a microscope**. The new PDMI-2 unit is heated and/or cooled by Peltier devices. It operates as an open perfusion chamber and features optical and micro-mechanical access. Perfusants at room temperature are brought to a temperature with in the microincubator itself temperature can be maintained from 8 °C to 46 °C (± 0.2 °C) for static conditions or with flow rates up to 3.0 min. A separate gas flow inlet provides temperature controlled gas across the chamber for temperature uniformity and pH control. Two chamber options are available: disposable 35 mm Petri dishes or the company's line of reusable cover

slip dishes. With a cover slip dish, high magnification (100 $\times$ objective), oil immersion, phase and interference contrast and fluorescence microscopy can be used. There are also two companion temperature controllers available: the first if heating only is required and the second if heating and cooling functions are needed. With either, electrical current is controlled proportionally to allow noise-free electrical recording.

Reader Service No. 107

Cell biology

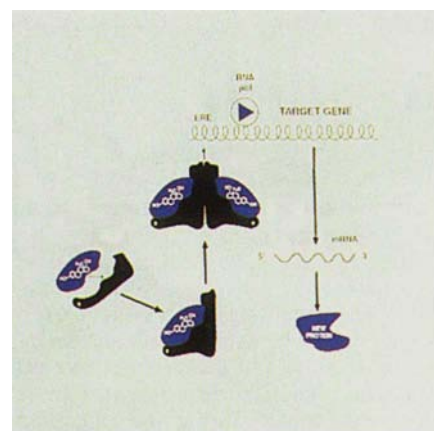
Organon Teknika now offers a new **catalogue** that includes 1,100 Cappel products and features 125 new primary antibodies. Primary antibodies are available to leukocyte markers, intermediate-filament proteins, extracellular-matrix proteins, cell-adhesion molecules, contractile proteins, oncogene proteins, nuclear antigens, autoimmune antigens, apolipoproteins, fusion-protein tags and infectious microorganisms. These antibodies were developed for use in immunoblotting and immunostaining of cells and tissue sections. Some antibodies, in addition, are suitable for use in immunoprecipitation and ELISA procedures. The catalogue is accompanied by a product listing with the prices of all Cappel products and custom antibody services.

Reader Service No. 108

BioResearch Ireland's National Cell and Tissue Culture Centre has an extensive **multidrug resistance (MDR) research programme** in progress. The company now offers two highly specific MDR monoclonal antibodies: BRI MAB MDR-1 [clone 6/1C] and BRI MAB TOPO II α [clone 4/2D]. Antibody 6/1C is supplied in a vial as undiluted ascites and contains 0.25 ml of murine monoclonal antibody. It is intended for use in immunoblotting (western blotting) and immunocytochemistry on cytospins/ cryostats and paraffin-embedded sections. This IgG1 isotype recognizes p-170 MDR and when used with the recommended protocols the volume provided should be sufficient for ten western blots and 125 immunocytochemistry stainings. For atypical MDR, for example non-p-170 MDR, the company offers antibody 4/2D — also supplied as undiluted ascites and provided in a vial containing 0.25 ml of murine monoclonal antibody. It can be used for western blotting, immunocytochemistry and for use on paraffin-embedded cells and sections. When used with the recommended protocols the volume provided is sufficient for 3 to 5 western blots and 130 immunocytochemistry stainings.

Reader Service No. 109

PanVera has announced the availability of **recombinant human oestrogen receptor**, a 67K member of the steroid-receptor



Pan Vera's human oestrogen receptor.

superfamily of transcription factors. The oestrogen receptor can act either positively or negatively in regulating expression of different genes involved in tissue growth and differentiation. When oestradiol is bound to the oestrogen receptor a series of events is initiated, including a conformational change of the ligand-binding domain, dimerization, binding to oestrogen response elements within target genes, and the stimulation of transcription. A baculovirus-mediated expression system is utilized to overexpress wild-type oestrogen receptor in infected insect cells.

Reader Service No. 110

Anti-Ash/Grb-2 monoclonal antibody and anti-MAP kinase monoclonal antibody Ash/Grb-2 are believed to play a role in signalling pathways between receptor protein tyrosine kinases and a Ras protein. Kamiya Biomedical's new anti-Ash/Grb-2 monoclonal recognizes an epitope in the amino-terminal SH₃ domain of human, rat, or mouse Ash/Grb-2. MAP kinase is believed to be important in the growth factor-stimulated signalling pathway. MAP kinase kinase induces phosphorylation of the MAP kinase. New anti-MAP kinase monoclonal reacts specifically with human and rat MAPKK (MEK1) and does not react with MEK-2. The Anti-Ash/Grb-2 monoclonal and anti-MAP kinase monoclonal antibodies should have many important applications in signal transduction research.

Reader Service No. 111

Transfection tools

Eppendorf Scientific now offers new **electroporation cuvettes**. The cuvettes are produced under stringent quality assurance procedures — individually packaged and sterilized with γ -irradiation. Gap-width tolerances are set to within hundredths of a millimetre to assure reproducible results. The cuvettes can be used to electroporate prokaryotic and eukaryotic cells, and

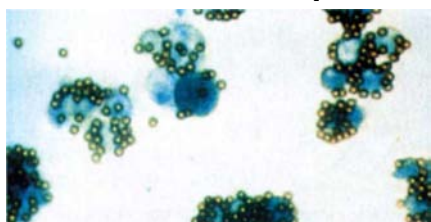
are available in three sizes (1-, 2-, and 4-mm gap width). Cuvette gap width and maximum volume are embossed on the side of the cuvette for easy size recognition. A frosted window permits cuvette labelling with marker or pencil. Eppendorf cuvettes are compatible with all common electroporation systems, for example Bio-Rad, BTX and Invitrogen. **Reader Service No. 113**



Electroporation cuvettes.

Transferrinfection is a new nucleic acid delivery system from Bender MedSystems that uses transferrin receptor-mediated endocytosis to carry DNA macromolecules into cells. **Receptor-mediated transfection** of eukaryotic cells is said to provide a gentle and efficient DNA transfection method that is based on a natural iron-uptake mechanism. Standard transfection protocols, by contrast, can often have deleterious effects on cell viability, Bender says. The application is simple, allowing Transferrinfection of DNA molecules from 3.5 kilobases to at least 15 kilobases with a similar efficiency. Transfection rates depend on the cell-surface expression of the transferrin receptor: cells with a high level of transferrin receptor expression (for example, human K562 and chicken HD3 cells from the erythroid lineage) show transfection rates of almost 100 per cent, according to the company. In other established cell lines, such as HeLa, CHO, COS and HepG2 cells, the method produces efficiencies comparable to other transfection techniques, but has the added advantage of being a gentle procedure. Two kit sizes are available with sufficient reagents for 30 to 50 transfections and 80 to 120 transfections, respectively. **Reader Service No. 112**

The Capture-Tec kit from Invitrogen allows researchers to **create a homogeneous population of transiently transfected cells without creating stable cell lines**. The kit features the pHook-1 plasmid, which expresses and displays a single-chain antibody to a specific hapten molecule. Users can simply co-transfect the plasmid and their construct, then isolate transfected cells with hapten-coated



Human breast carcinoma cells selected with Invitrogen's Capture-Tec beads.

magnetic beads in order to study intracellular events post-transfection. Selected cells may be used for differential display, western blots, flow cytometry, enzymatic analysis or pulse-chase experiments.

Reader Service No. 114

Molecular means

The GATC 1500 DNA analysis system uses the direct blotting electrophoresis (DBE) method, which combines electrophoresis and blotting. Single- or double-stranded DNA samples are simultaneously separated through conventional gels (agarose or polyacrylamide) and are blotted as they leave the gel onto a nylon membrane passing underneath the gel. In contrast to conventional DNA electrophoresis techniques, DBE results in the linear spacing of DNA fragments, thus improving the resolution of each run. Three separate DBE units are available to suit specific research requirements: the 'DNA Sequencer' offers sequence resolution of over 500 nucleotides; the 'Long Run DNA sequencer' is designed for single-run, 800-nucleotide resolution; and the multifunctional Navigator is optimized for PCR analysis, Southern and northern blotting, differential display, STR analysis, S_i mapping and up to 250-nucleotide sequencing resolution. Each unit comes as a stand-alone system and is ready for extension to any of the three GATC units.

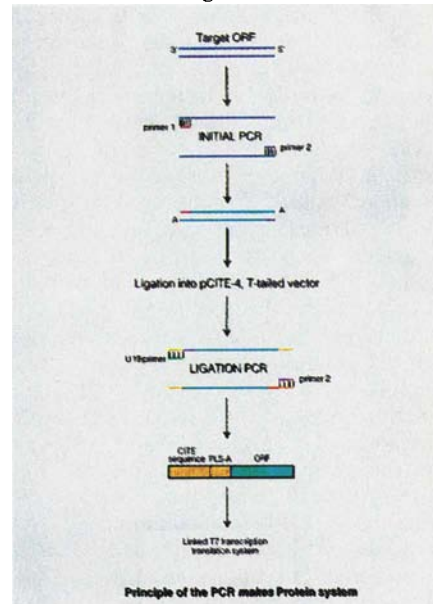
Reader Service No. 115

A new versatile **power supply for all electrophoresis applications** is now available from Hybaid. It offers a crossover facility, that automatically switches between current and voltage when a predetermined limit is reached. Another useful feature of the PS250 is the ability to retain output settings when the supply is switched off in the event of a power failure. This not only removes the need for resetting when power is returned, but also increases consistency between runs. Powerful enough to meet the demands of modern molecular biology laboratories, it can deliver up to 250 volts, 500 milliamps and 125 watts, working in either a constant current or constant voltage mode. The output is set through a large adjustment knob with a 24-hour, count-up/count-down timer, avoiding the need for frequent checks on the progress of a run. Safety aspects include a load-sensing circuit that shuts off the voltage if the lid is removed.

Reader Service No. 116

Amersham Life Science has introduced a range of new **transcription-translation kits for the *in vitro* production of proteins** directly from a variety of DNA templates, including plasmids (circular or linearized), and PCR templates con-

taining the T7/SP6 promoter. The Linked T7 and SP6 transcription-translation systems rely on a simple two-stage process, that first calls for a 15-minute transcription using premixed reagents to minimize pipetting errors and facilitate ease of handling. Amersham says salts are included in this transcription mix to avoid any need for optimization, even with suboptimal DNA concentrations. For translation, optimized rabbit reticulocyte lysate and a label (for example, Amersham's Redivue [³⁵S]methionine or ECL cell-free labelling system) are added directly to the transcription reaction. This two-stage format enables the



Amersham's *in vitro* protein production.

transcribed RNA to be isolated and analysed as desired. Alternatively, the 'PCR Makes Protein' system can be used to clone PCR templates into a T7 promoter-containing vector, avoiding the need to transform competent cells and perform minipreps. Using this system, the company estimates that at least one day can be saved, as compared with standard methods. With this system amplicons containing the open reading frame of interest can be ligated into the T-tailed vector, pCITE-4c(+), which is then subjected to further PCR. This vector incorporates a T7 promoter, avoiding the cost of incorporation into PCR primers, and a translational entry point giving a significantly improved protein yield. In addition, an in-frame stop codon ensures translation with no extraneous bands. The target sequence is then transcribed and translated using the linked transcription-translation system.

Reader Service No. 117

Users of *in situ* hybridization (ISH) may be interested to read that Boehringer Mannheim is now offering what it calls a **fast and sensitive alternative to tradi-**

tional ISH. The Primed *In Situ* labelling (PRINS) reaction kit is said to provide good signals from repeated DNA sequences in less than an hour, and from unique sequences in less than 3 hours; this compared with the overnight incubation required by other methods. Multiple sequential PRINS reactions are possible, just as in multiple FISH (fluorescence *in situ* hybridization) probing, by using different probes labelled with differently labelled nucleotides and visualized using various fluorescence reporter systems. However, with the PRINS labelling reactions, discrete sequential steps are performed and so the need for every probe to have a similar melting temperature is eliminated. This is said to increase sensitivity as hybridization is always performed at optimal stringency for each particular probe. The kit hybridizes an unlabelled synthetic oligonucleotide probe to the complementary nucleic acid sequence and utilizes elevated temperatures in combination with thermostable enzymes, such as *Taq* polymerase, to extend the hybridized primer in a single thermal cycle. In traditional ISH, the choice lies between short probes — which penetrate well and hybridize rapidly — or long probes containing greater amount of label, but with impaired penetration and slower hybridization. In PRINS, however, the extent of chain elongation is said to be unaffected by primer length and the strand of labelled nucleic acid is much longer than the probes themselves. Also, the short fragments penetrate well and offer fast hybridization kinetics during the shortened incubation. The result, says the company, is enhanced sensitivity in most applications. The background is also said to be inherently low, due to the use of unlabelled primers.

Reader Service No. 118

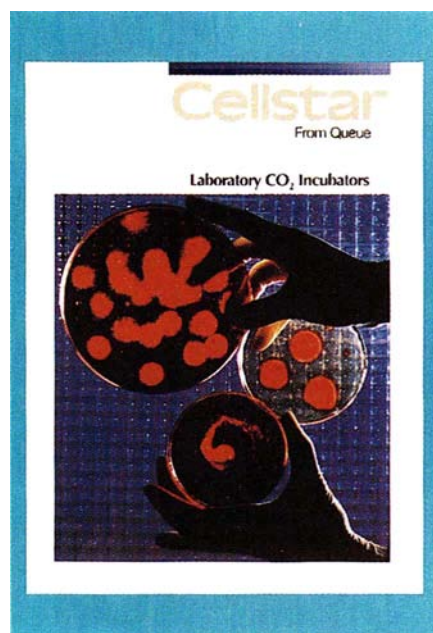
Culture and Incubation

The new B15 compact incubator from Heraeus has been designed to have an advanced ergonomic design and to require a minimum benchtop space. It should prove useful for a wide range of applications in any laboratory where incubating, tempering, thermal storage and drying are routine processes. It has a capacity of 15 litres and the operational temperature range of 20 °C to 50 °C, with a temperature deviation of 0.2 °C. An air-circulation fan ensures an even distribution of temperature throughout the incubator. The unit is fitted with a temperature protection system and will switch off automatically as soon as the temperature reaches 70 °C. Supplied as standard with a transparent, tinted cover to permit viewing of specimens, an opaque cover is also available for those applications that need to

exclude light from the chamber. The hinged cover opens upwards and a removable tray makes for easy loading and unloading of specimens. All parts of the incubator are fabricated from plastics that will withstand sterilization by standard disinfectants.

Reader Service No. 119

Queue Systems has issued a full-colour, 12-page brochure highlighting its new line of Cellstar laboratory CO₂ incubators for critical cell-culture applications. The new line includes models with specially designed features to enhance climate control and to provide added security. Included among these are a tri-gas sys-



Queue Systems brochure covering the company's new line of CO₂ incubators.

tem, thermal and infrared CO₂ sensing, a water- and air-jacketed design, Multiplex microprocessor environment control, a deep-drawn seamless interior construction and single or double chambering.

Reader Service No. 120

The Biostat A laboratory fermenter modules from B Braun Biotech can be configured to meet customer requirements. A range of autoclavable culture vessels (with 2-, 5- and 10-litre working volumes) and accessories and sensors is available, as well as peripheral equipment, such as rotameters, pumps, massflow controllers and heating blankets. All necessary measurements and controls are performed by the Micro DCU, digital control unit, which can be directly connected to the sensors and actuators, as well as to existing lab equipment. For data recording, the control unit is equipped with interfaces for recorder, printer and host computer.

Reader Service No. 121

BioWhittaker offers a bioreactor cell-culture system based on a hollow-fibre, within-fibre design, which are guaranteed autoclavable for five cycles. The manufacturer states that the bioreactor design provides near tissue cell density, high cell viability and high product yield. The non-membrane Secor-S oxygenator solves the problems of incorrect oxygenation. The MAbMax 200 and 400 series systems are equipped with Tricentric hollow fibre bioreactors the within fibre design.

Reader Service No. 122

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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- BRI MAB Topo-II oc [Clone 4/2D]
- BRI MAB LRP [Clone 3B6]

Extensive MDR research programme in progress

- Briclone Hybridoma Cloning
Medium also available



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